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THE UNIVERSITY OF ALBERTA

MORPHOLOGY AND FUNCTION OF ISLETS OF LANGERHANS SUBJECTED TO WARM ISCHEMIA

by

© HUGH LEWIS STEWART

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

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DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

FALL, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled MORPHOLOGY AND FUNCTION OF ISLETS OF LANGERHANS SUBJECTED TO WARM ISCHEMIA submitted by HUGH LEWIS STEWART in partial fulfilment of the requirements for the degree of Master of Science.

.....
Supervisor

.....

.....

Date October 5, 1976

To Dorothy and Dylan, who have sacrificed so much towards the completion of this thesis.

ABSTRACT

The temporal aspects of the adverse effects of *in situ* post-mortem warm ischemia on islet of Langerhans integrity were examined by histology, electron microscopy, and *in vitro* functional studies. Light microscopy was not informative as no definite morphologic changes were observed during up to 24 hours of ischemia. By electron microscopy, irreversible cell damage defined by the disruption of cell and organelle limiting membranes was noted by six hours. *In vitro* insulin secretion by isolated perifused islets was moderately impaired by 1/2 hour and severely reduced following one hour of ischemia, suggesting a similar degree of impairment of viability. Also, significant progressive decrements occurred in the number of islets isolated at 1/2 and one hour of ischemia and virtually no islets could be isolated beyond one hour.

Attempts to improve islet viability following ischemia by pre-treatment of the donor animal with methylprednisolone [30 mg/kg intravenously for 2 hours] produced a reduction in insulin secretion from control and 1/2 hour ischemic islets. This effect, which may have been secondary to the glucocorticoid actions of the drug, prevented any conclusions regarding its effect on viability of ischemic islets. Methylprednisolone did result in increased numbers of islets being harvested following one hour of ischemia but did not prolong the ischemic interval during which islets could be isolated.

The major conclusion of this research was that warm ischemia results in a rapid decline in yield and viability of islets of Langerhans. Accepting declining numbers and viability, islets for transplantation could be harvested from cadaver donors following warm ischemia up to a maximum period of one hour.

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CHAPTER I

I. INTRODUCTION

A. Research Aim

The objective of this research was to temporally delineate the adverse effects of warm ischemia [Appendix I] on the structure and function of islets of Langerhans. The practical application of this very basic information would be to provide guidelines for the harvesting of isolated islets for transplantation from human donors subject to ante-mortem ischemic insults or in the early post-mortem period.

B. Current Status of Islet of Langerhans Transplantation

Diabetes mellitus is an incurable disease with current medical knowledge. The momentous discovery of insulin has allowed for regulation of the metabolism of diabetic patients to achieve increased longevity and decreased morbidity. However, present therapeutic measures of diet, oral hypoglycemic agents, and exogenous insulin injections, no matter how complex or rigidly adhered to, cannot be the ultimate answers in this disease. The reasons for this are that they cannot completely normalize metabolism, particularly of glucose, and they do not prevent the appearance of numerous complications associated with diabetes. Whether these secondary complications arise from the inability of exogenous insulin to completely reverse the metabolic abnormalities or are due to the lack of other essential metabolic

substances in diabetic patients is not known. It is for these two reasons that alternative means of controlling and hopefully eventually curing this disease continue to be sought.

Not surprisingly in this era of avid interest in tissue transplantation, the possibility of restoring the diabetic patient's metabolic status quo by transplantation has been broached. The obstacle to this approach has been that the required endocrine tissue, the islets of Langerhans, which contain the insulin - producing beta cells and other hormone synthesizing cells, constitutes only 1-2% of the pancreas and is randomly located within the much larger exocrine component. Reducing the amount of exocrine tissue through atrophy by previous ligation of the major pancreatic ducts is obviously not applicable for human transplantation. Continuing attempts are being made to transplant the entire pancreas, or a major portion of it, to obtain the benefits of its minor endocrine component (19, 32, 51). These procedures are very difficult technically and meet not only with the expected rejection phenomena but also are fraught with complications arising from the superfluous exocrine portion of the gland and the often attached duodenum. Although the clinical experience with pancreatic grafts has been generally unrewarding, many of the recipients have had an interval of normalization of their glucose metabolism, some even to the point of no longer requiring insulin therapy, supporting the concept that transplantation of the endocrine portion is a valid therapeutic consideration. Therefore, techniques for transplanting isolated islets by themselves are being investigated.

It was documented early in this century by Bensley (7) that individual islets of Langerhans could be isolated from the pancreas. However, it was not until 1964 when Hellerstrom (38) was able to micro-dissect appreciable numbers of metabolically intact islets from several mammalian species that their possible use for transplantation came to be realized. During the ensuring decade considerable progress has been made toward elucidating the therapeutic potential of this method.

The continuing fundamental challenge of this technique has been to be able to harvest sufficient numbers of viable islets from one donor pancreas to adequately meet the insulin-production requirements of a diabetic recipient. In 1965, Moskalewski (63) provided the basis for current harvest techniques by using enzymatic digestion with collagenase to separate the exocrine acinar tissue from intact islets, allowing them to be hand picked from among the acinar debris. This method was improved upon by Lacy and Kostianovsky in 1967 (47) when they introduced the concept of mechanically disrupting the acinar tissue *in situ* by injecting a balanced salt solution via the pancreatic duct system and demonstrated that the islets could be separated from the collagenase-digested exocrine tissue by centrifugation in a discontinuous density gradient. With experience, approximately two hundred islets can be gleaned from one rat pancreas with this method (2) and an experimentally diabetic rat can be cured by the isogenic intraportal transplantation of 400-600 islets (44). Similar relative numbers of islets have been obtained from the pancreas of other species including man (81). The basic fault in this technique has

been that only those islets isolated at the time the digestion process is terminated are available for harvesting. Those separated earlier will have been disrupted by on-going enzyme digestion and those still attached to acinar tissue cannot be isolated. Recently, Scharp *et al* (74) have partially solved this problem. In their method, the pancreatic tissue to be digested is transferred into a cylinder made of stainless steel mesh with a pore size of 260 micron diameter, which is a suitable size to allow separated islets to pass out of the cylinder while retaining larger tissue fragments for further digestion. This apparatus is placed within a plexiglass tube for the digestion procedure which involves repeating sequences of adding collagenase solutions, agitating the vessel in a 37°C waterbath, and washing out the products of digestion at frequent intervals followed by density gradient centrifugation to obtain the islets separated at different times throughout the digestion process. This method has increased the number of islets recovered per rat pancreas to 450 and the islets from one donor pancreas can significantly ameliorate a recipient's diabetic state. Similar success has been achieved in isolating and transplanting islets from the pancreases of monkeys with this technique.

Considerable experience has been obtained in the transplantation of isolated islets in animals with experimental diabetes. These investigations have shown that the most efficient site for their administration is into the liver via injection into the portal vein (3, 44). Histologically, the islets have been shown to lodge in the terminal portal venules and become revascularized. With this technique, complete normalization of the clinical parameters of diabetic monitoring -

body weight, blood sugar, urine volume and glucose content - can be achieved. However, oral and intravenous glucose tolerance tests tend to evoke mildly diabetic patterns of insulin release and glucose metabolism (70, 72). The cause of this persistent abnormality has yet to be explained but speculation has suggested such factors as inadequate numbers of transplanted islets, the autonomic denervation of the grafts, or the abnormal anatomic location of the islets within the portal venous system.

Some data has been presented relative to the fundamental question of whether islet transplantation can prevent the emergence or progression of the serious secondary complications of diabetes. Ziegler *et al* (88) have demonstrated normalization of elevated serum cholesterol and triglycerides in streptozotocin-diabetic rats following isografts. Mauer *et al* (57, 58) have shown that the early renal glomerular lesions in experimental diabetes in rats, consisting of histopathologic mesangial thickening plus immunohistochemical demonstrations of depositions of IgG, IgM, and complement within the mesangium, can be reversed, or their progression halted, by islet transplantation. However, it is debatable whether the morphologic and metabolic abnormalities in animals with chemically-induced hyperglycemia are the equivalent of human diabetic pathology.

The transplantation immunology of isolated islets of Langerhans has also been investigated. From early work with whole pancreas homo transplants, histologic and functional evaluation of the islets suggested that they were relatively spared by the rejection process, giving rise to hopes that they might be an immunologically privileged

organ (75). However, as experience with non-immunosuppressed allogeneic pancreatic transplantation has accumulated, the islets have been shown to obey all the rules of transplantation immunology and their pattern of rejection is similar to that of the exocrine tissue with a mean survival time of 8-12 days (1, 41). Surprisingly, when isolated islets are transplanted across strong histoincompatibility barriers and their survival assessed by their ability to normalize glucose metabolism, they appear to undergo a very rapid first set rejection with a mean survival time of 1-3 days (71, 88). It is difficult to explain such a rapid loss of function of the grafts as being due to immunologic attack. These observations can probably be more appropriately explained from analysis of the pattern of glucose metabolism after isogeneic islet transplantation. Upon isografting, an abrupt decline in blood and urine glucose levels of 1-3 days duration occurs with a subsequent return to a more diabetic state followed by a gradual improvement in glucose homeostasis over approximately one week (50). The initial response is probably due to the passive release of insulin from damaged islets superimposed on disturbed feeding habits resulting from the operation while the more slowly evolving resolution of the diabetic state represents true physiologic function of the islets. In allogeneic transplantation, the transient apparent improvement in glucose regulation occurs but the permanent functional establishment of the islets over the next few days is interrupted by rejection phenomena occurring at the usual time. Attempts to prolong the survival of allografted islets with immunosuppressive agents have shown some benefit from azothioprine (2) and antilymphocyte serum (88) but the results to date

have been unspectacular.

The answers to the questions of whether islet transplantation can favorably influence diabetic complications and whether prolonged survival and function of the grafts can be achieved must await transplantation into human diabetics. At least one clinical trial is currently in progress (64) but widespread implementation of islet transplantation must be considered to be premature and not condoned at this time (33, 65). The major deterrant to human transplantation studies is the fact that immunosuppressive therapy would naturally be necessary. The side effects of the present methods of preventing rejection plus the high risk of infection in immunosuppressed diabetics are not safe alternatives to the current means of treatment in the majority of cases.

It has perhaps been fortuitous that pancreatic and isolated islet transplantation have been difficult problems in that the precocious clinical introduction of such therapeutic means as occurred with other organs has been prevented. Now that human isolated islet transplantation is beginning to be employed the fundamental knowledge of the technique is extensive.

C. Rationale for Research Project

Should islet of Langerhans transplantation prove successful in preventing the evolution of the complications of diabetes mellitus, it would become a vitally important preventive health measure. Transplantation would be indicated not just in a small group of patients with advanced secondary lesions but perhaps in the entire population of diabetics. However, could such widespread application of this therapy

ever be possible?

From the accumulated experience with kidney transplantation in the relatively small number of renal failure patients, it has become apparent that the availability of donor tissue is a major limiting factor in meeting the demands for transplantation (25, 66). The shortage of organs is due to the limited number of suitable donors plus the appreciable percentage of harvested kidneys that must be discarded or fail to function after transplantation due to warm ischemic damage. The first problem can be dealt with only through expanded public relations programs in hope that public awareness will increase the number of organ donations. Continued scientific investigation can contribute to a more thorough understanding of the pathophysiology of warm ischemic injury and discover more effective methods by which it can be minimized.

In human islet of Langerhans transplantation the short supply of donor tissue can be expected to strictly limit the number of patients to be treated. Islet transplantation requires their isolation using harvest procedures which to date have poor percentage yields. In contrast to renal transplantation where one donor can provide organs for the treatment of two patients, islet transplantation may require multiple donors per recipient. Also, as islets are situated in an unpaired organ, living donors, who have been a valuable source of kidneys for transplantation in some countries, will not be able to be used. If the number of donors and the percentage of islets isolated from each pancreas cannot be increased, then the maximum viability of tissue from all potential donors becomes a critical factor if islet

transplantation is going to be more than a rare occurrence. It is for this reason that efforts should be made to determine the period of ischemia that islets can tolerate while maintaining structural and functional integrity.

There are several reasons why it would seem that the parameters established for the temporal course of warm ischemic injury in other transplantable organs, most notably the kidney (18, 87) and liver (27, 30) cannot be applied directly to the islets of Langerhans. Islet function is not based on complex cellular organization as is present in the kidney where the tubular epithelium is most sensitive to ischemia (87). Islet transplantation does not require direct revascularization by surgical anastomoses with the resultant disturbed hemodynamics secondary to ischemic injury (59, 82). Also, islets do not manifest organ failure to the degree that occurs in the kidney following other ischemic insults such as hypotension. Hypoinsulinism and hyperglycemia do occur during shock states but these changes are of multifactorial and as yet incompletely defined etiology (16, 22). It is postulated that the inappropriate reduction in serum insulin levels may be due to beta cell unresponsiveness secondary to hypoxia or inhibition of insulin release by the sympathetic nervous system response to stress. The hypoinsulinism occurs in spite of a reduced rate of insulin degradation (16). Whatever the cause, these alterations in the homeostatic mechanisms for glucose metabolism are only transient phenomena in those subjects who recover from shock (13). These idiosyncrasies would appear to justify the investigation of the effects of warm ischemia on the subsequent viability of islets of Langerhans for transplantation.

CHAPTER II

MORPHOLOGY OF ISLETS OF LANGERHANS FOLLOWING EXPOSURE TO WARM ISCHEMIA

To obtain a perspective of the temporal course of ischemic damage to islets, their morphologic integrity following progressively prolonged periods of exposure to warm ischemia *in situ* was examined histologically and by electron microscopy. Any structural deterioration that could be observed during the study intervals was expected to be an indication of viability following ischemic insults and to provide a time reference on which to plan subsequent functional studies.

A. Materials and Methods

All specimens were obtained from non-fasted male Wistar rats weighing 300-350 grams. Control animals had pancreatic tissue removed under ether anesthesia. Individual study animals had tissue specimens collected at 1, 2, 4, 6, 12, or 24 hours after being sacrificed by over exposure to ether and kept at room temperature [22-26°C]. Two series of animals were studied, one by light microscopy and the other by electron microscopy, utilizing the following techniques.

1. Histology

Total pancreatectomy was performed and the entire specimen was immediately placed in Bouin's fluid [picric acid, saturated aqueous solution, 750 ml; 40% formaldehyde, 250 ml; and glacial acetic acid, 50 ml] (76) for at least 6 hours to allow for tissue fixation. Blocks approximately 0.5 cm square were excised from the specimen, dehydrated

in a graded series of alcohol, and embedded in paraffin. Sections 5-6 microns thick were cut on a microtome. These were stained with a modification (77) of Gomori's aldehyde fuchsin (34) which is specific for the secretory granules of the beta cell component of the islets, and counterstained with metanil yellow (77).

2. Electron Microscopy

Each specimen consisted of ten 1×2×2mm [approx.] pieces of tissue sharply excised from random areas of the pancreas. These were transferred immediately to 3.5 ml 6.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.3, at 4°C and kept at 4°C for a minimum of 24 hours to allow for fixation of the tissue. They were then washed overnight in three changes of cacodylate buffer and post-fixed in osmium tetroxide (14) for two hours at 4°C. The tissue was dehydrated by rapid changes in graded alcohols followed by propylene oxide and was embedded in Epon 812 modified from Luft (53). Silver sections were cut on a Reichert OM-U2 Ultramicrotome equipped with a diamond knife and were placed on 400-mesh uncoated copper grids. Sections stained with uranyl acetate (80) followed by lead citrate (26) were viewed in a Siemens Elmiskop 1A electron microscope.

B. Results

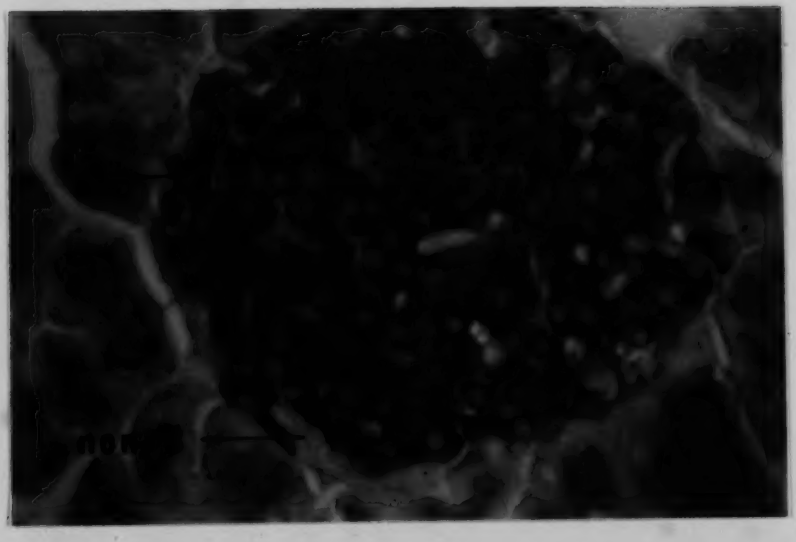
A normal islet of Langerhans is shown in Figure 1. The beta cells, whose secretory granules stain deep purple with aldehyde fuchsin, comprise the majority of the endocrine cells of the rat islet. A few cells with unstained granules present at the periphery of the islet represent the non-beta [alpha, delta, or c] endocrine cells. Although unencapsulated, the islets are demarcated from the surrounding

FIGURE 1. Normal rat islet of Langerhans [×400].

B - beta cell

non-B - non-beta endocrine cell

arrow - capillary



acinar tissue by a thin layer of reticular tissue (8). They possess a rich capillary supply to provide for rapid exchange of secretagogues and hormones.

Islets subjected to 12 and 24 hours of warm ischemia *in situ* are shown in Figures 2 and 3. Although rather prominent histologic processing artifacts are present, the overall architecture of the islets apparently was preserved. The beta cells appeared to have maintained their integrity and still contained abundant secretory granules. Nuclei were present within the cells but appeared pyknotic, although lack of chromatin staining made this difficult to confirm. Similar normal appearing structure of the islets with no definite evidence of irreversible cell damage was observed at all time intervals studied. Rather than employ more diverse histologic techniques to determine if any morphologic deterioration was occurring during the study period, electron microscopy was undertaken.

The ultrastructural appearance of a normal rat islet of Langerhans (40, 69) is depicted in Plate I. The endocrine cell types are distinguishable by the charactersitic morphology of their secretory granules. The beta cells have granules of two types: those with an electron dense irregularly spherical core separated from its limiting membrane by a relatively wide zone of low density plus others containing pale amorphous granules within a more tightly fitting membrane. The alpha cell granules have a variable high density central core with a narrow medium density peripheral halo to which the limiting membrane is closely applied. The other intracellular organelles - notably the mitochondria, golgi apparatus, and endoplasmic reticulum - appeared

FIGURE 2. Rat islet - 12 hours warm ischemia [×400].

FIGURE 3. Rat islet - 24 hours warm ischemia [×400].

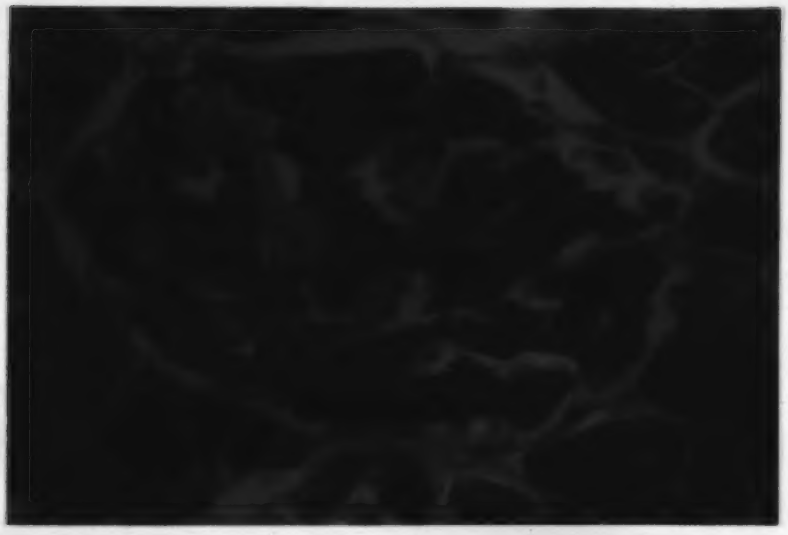
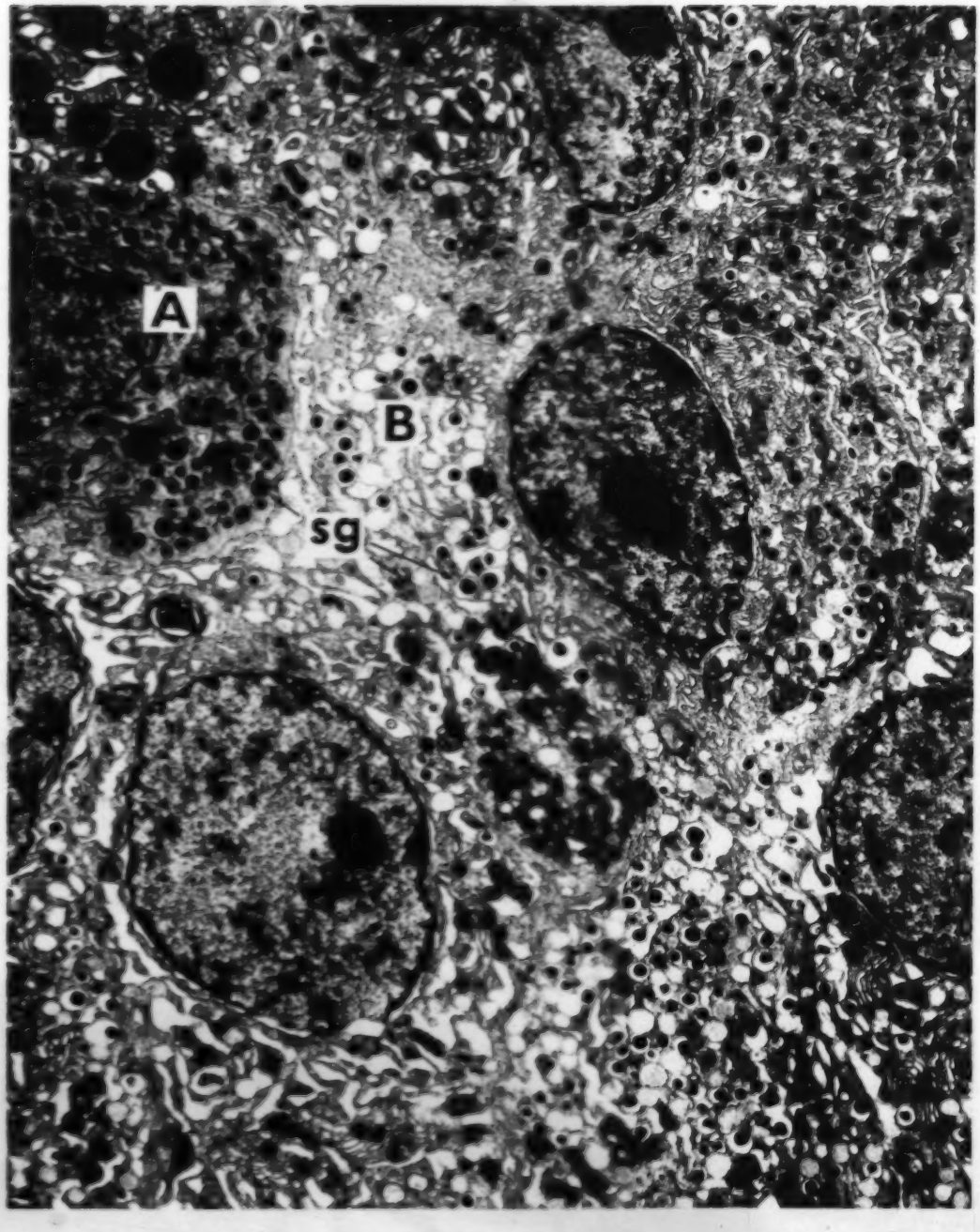


PLATE I. Ultrastructure of rat islet of Langerhans, normal. [×11,000]

A - alpha cell

B - beta cell

sg - secretory granules


A
B
sg

quite normal in this preparation. The nuclear chromatin clumping was due to glutaraldehyde fixation (83).

Following exposure to warm ischemia *in situ*, the ultrastructural appearances varied somewhat from cell to cell at each time interval. However, from the series of specimens studied, a sequence of events for the general population of endocrine cells could be described as follows.

No islet tissue could be found in the tissue from the rat subject to one hour of ischemia.

After two hours ischemia [Plate II] there was evidence of a moderate degree of cell injury (83). Some mitochondria were in a condensed de-energized phase while the majority demonstrated mild to moderate high amplitude swelling. There was widening of the less dense peripheral halos of the secretory granules, particularly in the beta cells; a mild degree of widening of the lamellae of the endoplasmic reticulum; and commencing swelling of the cytoplasmic ground substance, especially peripherally, giving the appearance of the organelles being concentrated toward the centre of the cells. These organelle and cytoplasmic changes indicate electrolyte and water shifts occurring due to disruption of membrane transport mechanisms secondary to diminishing intracellular energy sources, notably ATP (6, 83). Early nuclear chromatolysis had occurred. An occasional "dark cell" was also seen, suggesting dehydration of the cell secondary to injury.

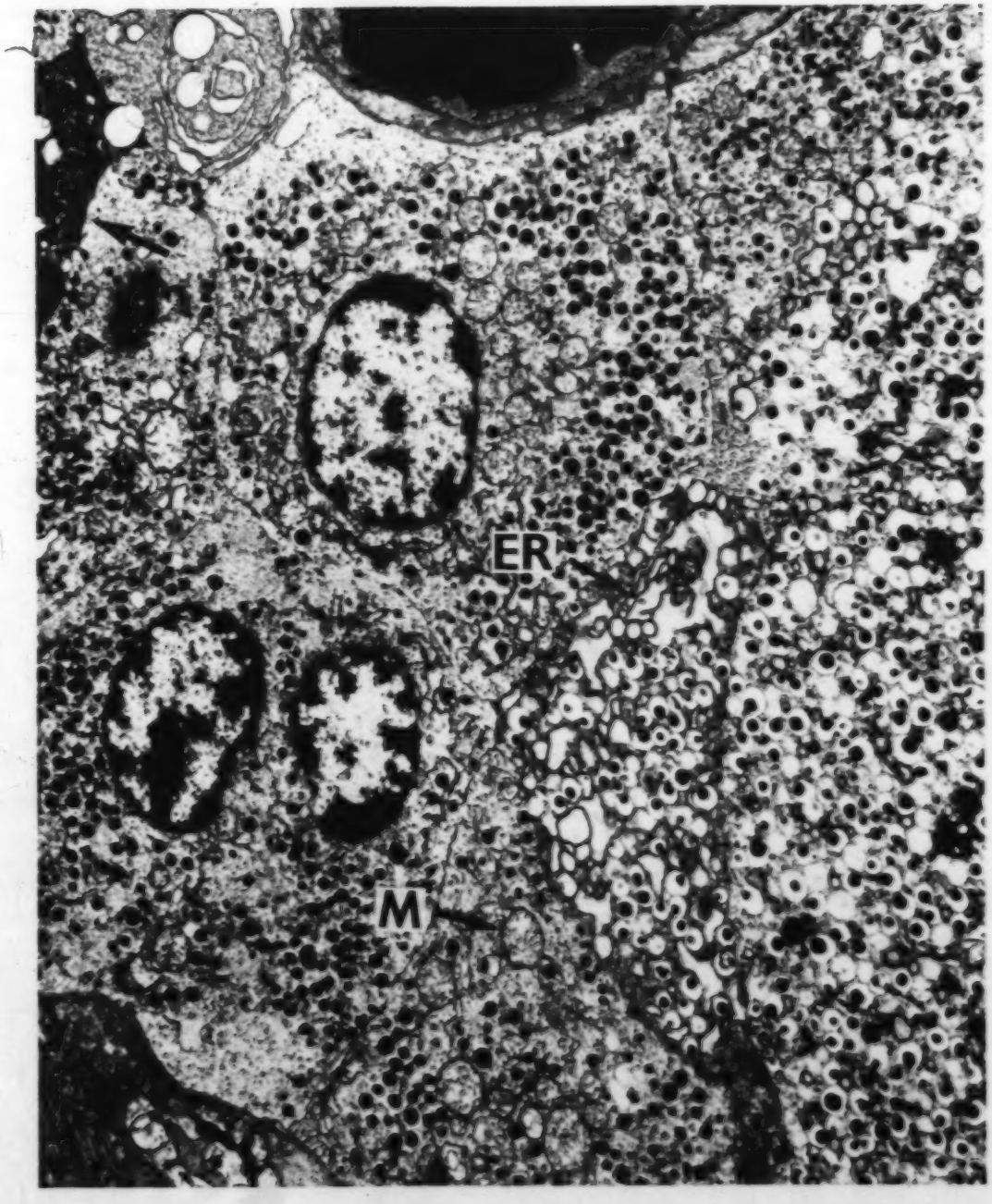
At four hours the demonstrated changes were similar to, but slightly more severe than, those observed after two hours. The main features were progressive dilation of the granules limiting membrane, making differentiation between cell types difficult, and further

PLATE II. Ultrastructure rat islet, two hours warm ischemia. [×9000]

ER - endoplasmic reticulum

M - mitochondrion

heavy arrow - dark cell


ER
M

swelling of the endoplasmic reticulum with vesicle formation [Plate III].

By six hours [Plate IV] progression of the above changes was associated with considerable swelling of the cellular ground substance and a greater degree of nuclear chromatolysis. More importantly, the disruption of both cell and organelle limiting membranes at that time can be accepted as the first definite indication that irreversible structural damage had occurred.

After twelve hours of warm ischemia [Plate V] there was almost total disintegration of the ultrastructure of the islets, the only means of identifying them being the persistence of small numbers of secretory granules. The intercellular spaces contained large amounts of cellular debris. Numerous intracellular and occasional extracellular myelin figures were present.

In view of the positive evidence of irreversible cell damage at six hours and severe disruption of the islets at the subcellular level by twelve hours, the 24-hour specimens were not examined.

C. Discussion

The preservation of integrity of the ischemic islets of Langerhans observed with the resolving powers of the light microscope indicate that this was an insensitive technique, when compared with electron microscopy, for determining the morphologic end-point of cell survival. There were no varying parameters by which a time of irreversible injury could be determined. Therefore, histologic methods would seem to be of little value in studies assessing the viability of islets for transplantation.

The ultrastructural changes demonstrated in islets exposed to warm ischemia are in no way unique to this organ. Similar alterations

PLATE III. Ultrastructure rat islet, four hours warm ischemia. [×9000]

ER - endoplasmic reticulum

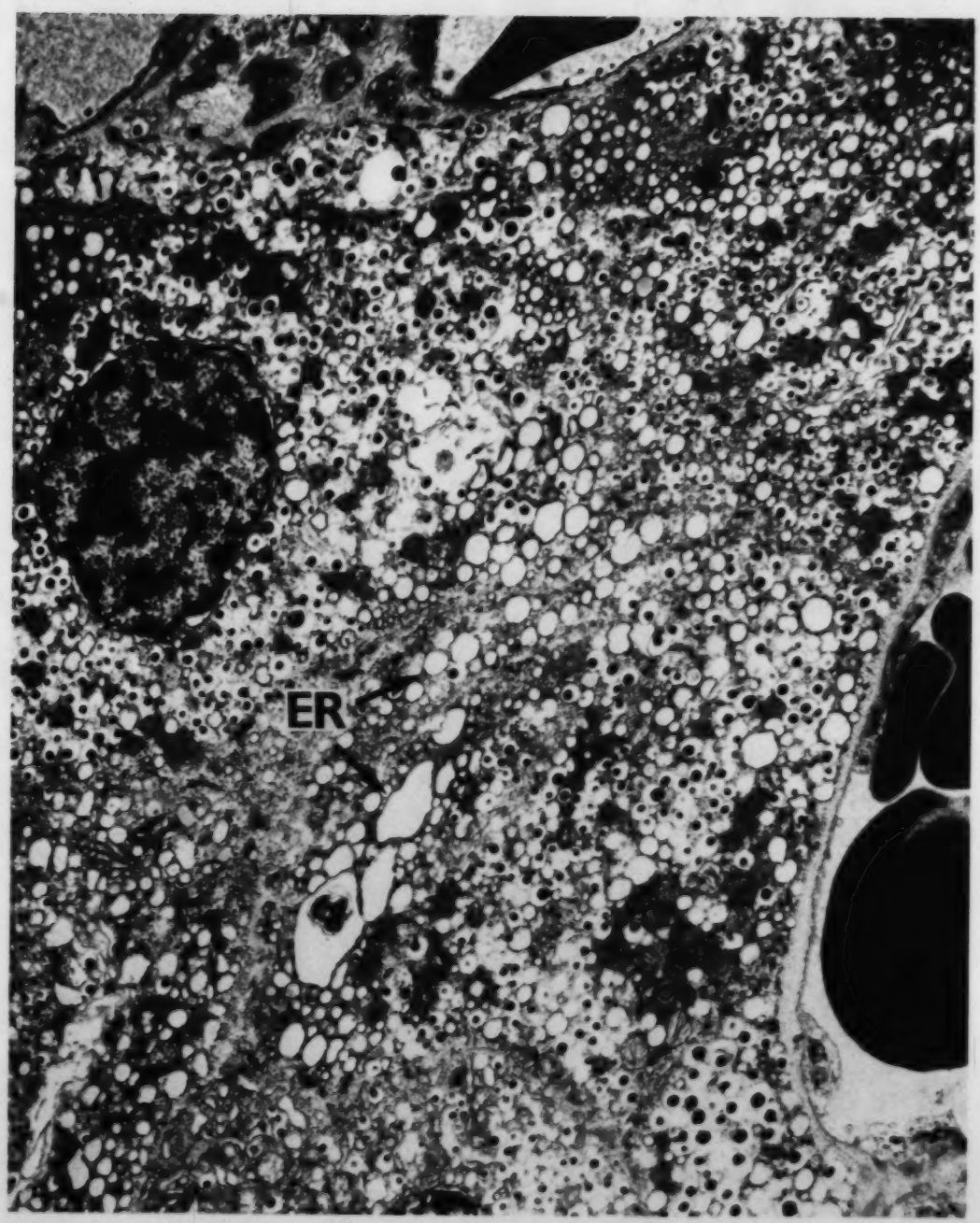

ER

PLATE IV. Ultrastructure rat islet, six hours warm ischemia. [×9000]

EX - exocrine cell

PM - plasma membrane

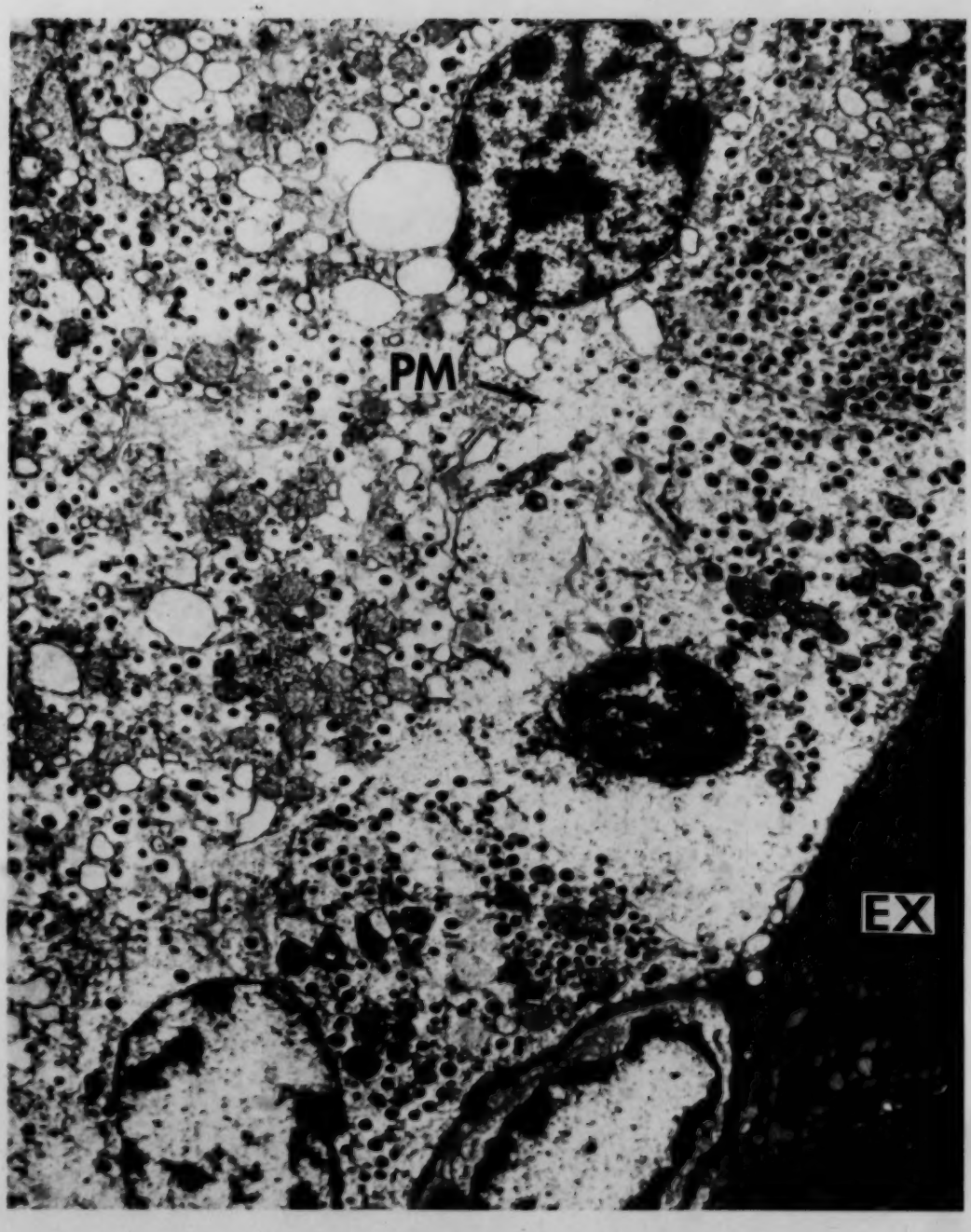

PM
EX

PLATE V. Ultrastructure rat islet, twelve hours warm ischemia. [×9000]

sg - secretory granules

mf - myelin figures

IS - intercellular space

EX - exocrine cell

mf
sg
IS
EX

occur in all cells subject to ischemic injury and during autolysis. The significance of the progression to irreversible damage observed in this study is the time sequence determined. That is, structural damage suggestive of cell death had occurred following six hours of exposure to warm ischemia, beyond which time progression to complete ultrastructural disorganization ensued. Therefore, by morphologic criteria, the maximum time interval during which islets of Langerhans can be harvested from post-mortem donors and be expected to retain potential viability is less than six hours. However, as lethal functional impairment usually has occurred prior to irreversible structural changes, the time criteria for viability of cadaver islets established by this phase of investigation may be too generous.

Similar ultrastructural studies of cellular degeneration during ischemia and autolysis have been reported for other transplantable organs, notably liver and kidney.

Bassi and Bernelli-Zazzera (5) studied morphologic deterioration and its reversibility in hepatocytes of rats subjected to up to 120 minutes of interruption of vascular supply plus following a two-hour recovery period of reperfusion. They noted early [15 minute] degeneration of the plasma membrane adjacent to the space of Disse, which they attributed to the lack of a buttressing effect of adjacent cells, associated with a large flux of plasma into the cells. Extensive disruption of the plasma membrane around the remainder of the cell was visualized at 120 minutes. Swelling of mitochondria and endoplasmic reticulum present at 30 minutes had progressed by 120 minutes to pronounced mitochondrial swelling and severe vesiculation and fragmentation of the

endoplasmic reticulum. The ischemic changes were less marked in fed animals with adequate hepatic glycogen stores in contrast to fasted subjects. Reperfusion of the cells for two hours reversed the structural alterations occurring with up to 60 minutes of ischemia but not those induced by 120 minutes, those cells going on to necrosis with the exception of some in the non-fasted animals.

Studying cadaver pig livers left *in situ* following sacrifice, Slagel and Eiseman (78) noted mitochondrial swelling, vesiculation of the endoplasmic reticulum, and occasional ruptures of the cell membranes adjacent to the space of Disse within 30 minutes. Mitochondrial membrane rupture, complete disorganization of the endoplasmic reticulum, and ruptures throughout the cell membrane were observed at one hour. They proposed that the structural disintegration correlated well with previously demonstrated reductions in metabolic activity [ATP generation, O_2-uptake, ammonium clearance, and bromsulphalein clearance] of a moderate degree at 30 minutes and severe at 60 minutes of ischemia (84).

Studies of renal ultrastructure during ischemia are complex due to the variable resistance of different components of the nephron to ischemia, most notably the sensitivity of the proximal convoluted tubule. Cook *et al* (20) and Latta *et al* (49) examined the entire nephron undergoing *in vitro* autolysis and confirmed that the degenerative changes were manifest most rapidly in the proximal convoluted tubule. The earliest abnormality [20 minutes] was swelling of the microvilli at the free brush border of the cell. By four hours dramatic changes of generalized swelling and obliteration of microvilli, cytoplasmic swelling, fragmentation and vesiculation of endoplasmic reticulum, mitochondrial swelling

and fragmentation, and nuclear shrinkage were observed. Beyond four hours there was progressive loss of the limiting membranes of the cell, some endoplasmic reticulum, and mitochondria. Similar morphologic deterioration developed more slowly throughout the remainder of the tubules and glomerulus.

Okada (67) demonstrated alterations in the proximal tubule cells of rats after two hours of renal pedicle clamping. The notable changes were marked distortion and swelling of the microvilli of the brush border and swelling of the mitochondria. These structural changes reversed slowly over approximately one month following resumption of circulation.

From these studies, it is apparent that ultrastructural deterioration occurs rapidly in hepatocytes subject to ischemia, irreversible destruction of cell and organelle morphology having occurred following periods of ischemia in excess of one hour. The morphologic changes described in the renal nephron in *in vitro* autolysis studies are more difficult to interpret due to the variable degree to which different levels of the nephron are affected with time but indicate that extensive and probably irreversible structural damage has occurred by four hours. Therefore, the less than six-hour time interval during which limiting membranes are maintained in islets of Langerhans subject to warm ischemia is considerably longer than in hepatocytes and perhaps longer than at least the proximal convoluted tubule cells of the kidney.

CHAPTER III

IN VITRO FUNCTION OF ISLETS OF LANGERHANS SUBJECTED TO WARM ISCHEMIA

The objective of the second phase of investigation was to establish a relationship between the duration of exposure to warm ischemia *in situ* and the impairment of *in vitro* function of islets of Langerhans isolated as for transplantation. This result would be an indication of their viability following such an insult.

A. Rationale for *In Vitro* Research Model

The specialized function of the beta cell component of the islets of Langerhans is to synthesize and secrete insulin to meet the body's homeostatic requirements, particularly for glucose metabolism. Evaluation of the insulin secretory response following exposure to insulinotropic agents is therefore a means of assessing the metabolism of the beta cell. From studies directed towards elucidating the mechanisms of insulin secretion, an *in vitro* method of investigating this response, a "perifusion" system, was devised.

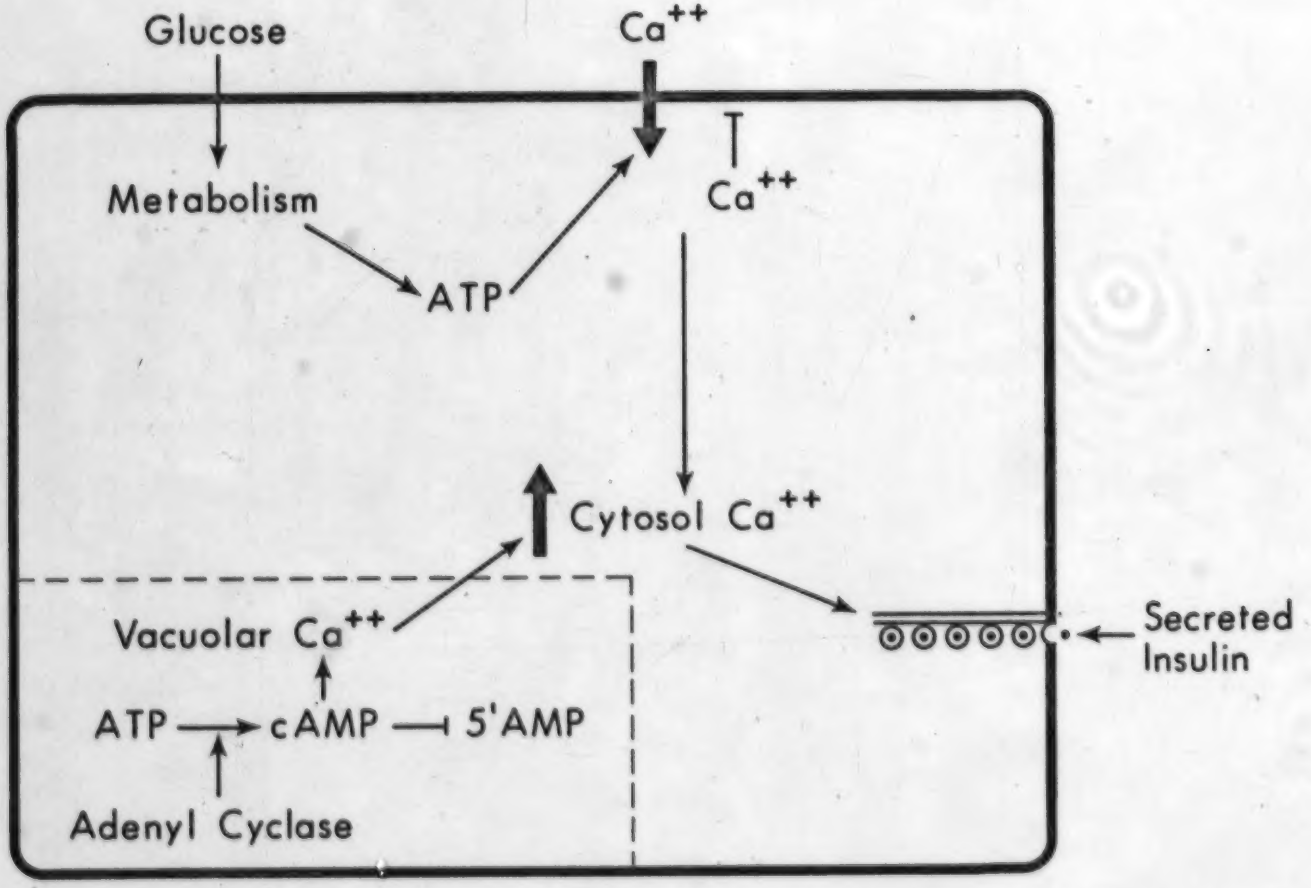
This perifusion method was first applied to isolated islet preparations by Lacy *et al* in 1972 (48). The islets are placed on a 5 micron pore size filter paper within a small filtration chamber through which a balanced salt solution with added albumin and glucose is continuously passed at a flow rate of approximately one millilter per minute. An initial stabilization period of at least 30 minutes of exposure to low glucose concentration [30 mg percent] allows insulin

release to diminish to baseline levels. To stimulate insulin secretion, the glucose concentration of the perifusate fluid is changed to 300 mg percent. Samples of the effluent fluid are collected at appropriate time intervals during perifusion and the amount of insulin secreted is determined by radioimmunoassay. Typically, normal islets secrete insulin in a biphasic pattern upon stimulation with elevated glucose concentrations. The early peak of release is thought to arise from a small rapidly mobilized insulin pool and is followed by a more prolonged phase in which the elevated rate of insulin secretion is from less labile stores and newly initiated synthesis. This response pattern is similar to the changes in serum insulin concentration following glucose stimulation in normal subjects *in vivo* (15). The technique has been used to investigate the dynamics of insulin secretion and the effects of various pharmacologic agents on this process.

The *in vitro* perifusion technique was considered to be a suitable model for attempting to temporally define the deleterious effects of warm ischemia upon islets of Langerhans integrity. The basis for this decision was that the parameter to be observed - insulin secretion - is an active cell process requiring intact metabolic pathways and therefore is an index of viability of the islets' beta cell population.

A model for the mechanism of insulin secretion [Fig. 4] was reviewed by Malaisse in 1975 (53). For insulin secretion to occur following glucose stimulation, glucose must be metabolized to generate ATP and perhaps be transformed into an intermediary metabolite which initiates insulin release. Blocking the phosphorylation of glucose by mannoheptulose administration inhibits insulin secretion (21, 55)

FIGURE 4. Mechanism of insulin secretion from beta cell.


Glucose
Ca++
Metabolism
Ca++
ATP
Cytosol Ca++
Vacuolar Ca++
Secreted
Insulin
ATP
cAMP
5'AMP
Adenyl Cyclase

but the metabolic pathway involved has not yet been determined. The ATP generated provides the energy requirements whereby calcium ions are accumulated within the cytosol through increased influx via a sodium-dependent pump mechanism plus inhibition of their efflux (54). These ions serve an intermediary function by inducing conformational changes in cell membrane-associated microtubular structures to which are attached insulin-containing granules (46). The secretory granules are thereby propelled to the cell surface and their insulin content released by emiocytosis (45, 68). The initial rapid phase of insulin secretion is accounted for by granules previously associated with the microtubules while the second phase represents granules free in the cytoplasm becoming attached to and secreted by the system (45). This glucose-sensitive pathway of insulin release is under the modulating effect on another active metabolic process - the cyclic AMP system (9, 62). Accumulation of cyclic AMP within beta cells, either by its increased rate of formation from ATP via adenyl cyclase or inhibition of its phosphodiesterase-mediated degradation, potentiates glucose stimulated insulin secretion. It is proposed that cyclic AMP causes an intra-cellular shift of calcium ions from within vacuoles to the cytosol, augmenting the stimulus-secretion coupling mechanisms for glucose. Disruption of these sophisticated energy-requiring processes by the pathophysiologic consequences of ischemia will impair the ability to secrete insulin. Such damage will be detectable in the secretory responses demonstrated by perifusion of isolated islets.

B. Materials and Methods

All experiments used nonfasted adult male Wistar rats. The following groups were studied:

Experimental Group	*Number of Experiments*
Control	10
1/2 hour Ischemia	10
1 hour Ischemia	10

The control group represents those animals in which the harvesting procedure was begun with the animals alive under ether anesthesia. The islets from these animals would be expected to have the optimum insulin-secretory potential to which all others may be compared. The animals in the ischemic study groups were sacrificed by an overexposure to ether and left at room temperature [22-26°C] for the desired time interval.

1. Isolation of Islets of Langerhans

The islets were harvested using a modification of the method of Lacy and Kostianovsky (47).

After shaving the animal's abdomen, a generous midline laparotomy incision was performed. The duodenum was mobilized to facilitate visualization of the course of the common bile duct. At the point where it enters the duodenal wall, the common bile duct was ligated with a 4-0 silk suture, one end of which was left long for traction. The superior portion of the common bile duct was then exposed just below the juncture of the hepatic ducts and, using blunt dissection, was skeletonized for approximately 0.5 cm. Just inferior to this region,

a 4-0 silk ligature was placed around the duct and tied loosely. Using fine scissors, the exposed duct was incised through approximately one-half of its diameter and a tapered polyethylene cannula of P.E. 10 calibre was introduced into the duct via this incision and secured in place by finishing the knot in the previously placed suture. Using a 5 ml disposable syringe, Hank's balanced salt solution [HBSS] at 4°C was injected through the cannula to distend the pancreas and disrupt the acinar tissue. The injection was continued until leakage of the solution through a rent in the pancreatic capsule occurred, usually after 15 to 20 ml of HBSS had been injected. The entire distended pancreas was then quickly excised and placed in a petri dish on ice containing 30 ml of HBSS.

The pancreas was rapidly minced with sharp scissors into fragments 1 to 3 mm in diameter. The tissue and solution were transferred to a 50 ml conical centrifuge tube on ice and allowed to stand 2 minutes to allow for sedimentation of the fragments. The supernatant was removed by suction and the tissue was washed twice with 30 ml volumes of cold HBSS, allowing 2 minutes for sedimentation of the tissue on each occasion. Following the final wash, there would always be 2.5 to 4.0 ml of minced pancreatic tissue. One millilitre of the final supernatant was left in the tube with the tissue and to it was added 16 mg of collagenase [Type 1, Sigma]. The tube and its contents were then transferred to a 37°C waterbath and agitated vigorously by hand to accomplish digestion of the acinar tissue from the islets of Langerhans. This digestion was continued until, by visual inspection, all the tissue fragments had been disrupted. This required approx-

imately 18 minutes of digestion for the freshly harvested control group and 2 to 3 minutes less for the ischemic tissue, suggesting some degree of autolysis in the latter. The digestion process was terminated by adding 40 ml cold HBSS to the tube and returning it to an ice bucket. This was followed by a 4-minute wait to allow the suspended tissue to sediment, following which the supernatant was aspirated off. The tissue was resuspended in 10 ml cold HBSS and decanted into a 15 ml conical-bottom centrifuge tube and again allowed to settle for 4 minutes. The supernatant was then removed as completely as possible by suction, leaving approximately 1 ml of finely digested tissue.

This tissue digest contains isolated islets plus contaminating acinar tissue which can be separated by discontinuous density gradient centrifugation. Clinical grade Dextran [Appendix II] of average molecular weight of 170,000 was dissolved in HBSS in concentrations of 30, 24, 18, and 10% and refrigerated at 4°C. The digested tissue was thoroughly resuspended in sufficient quantity of the 30% Dextran solution to make a volume of 5 ml. On top of this was layered 2 ml volumes of the other Dextran solutions in order of decreasing concentration. Centrifugation was performed at 800 g for 15 minutes. Inspection of the gradient after centrifugation revealed variable numbers of islets of Langerhans visible as small white specks at the interface of the 10 and 18% Dextran solutions with a greater number at the 18/24% interface. The preparation was usually contaminated with a few small lymph nodes at the 10/18% interface plus small amounts of ductal elements and acinar tissue at the 18/24% interface. These isolated islets could then be transferred to the perifusion apparatus described

below. This harvest procedure required approximately 90 minutes.

2. Perifusion of Isolated Islets of Langerhans

Perifusion of isolated islets of Langerhans was performed using a technique similar to that of Lacy *et al* (48).

The major component of the apparatus [Fig. 5] was a reusable plastic millipore filter chamber [Gelman] into which the isolated islets could be placed. The filter papers used were 24 mm in diameter with a pore size of 5 microns. To the outlet aperture of the chamber was attached to a 55 cm length of Silastic tubing [Dow Corning] of 2 mm internal diameter which was secured in a peristaltic pump calibrated to convey 1 ml of solution per minute. To the inlet was attached a 30 cm length of the same tubing to aspirate the perifusion fluids into the system. The volume of this system was approximately 3 ml and therefore a 3 minute interval was required for a change in the perifusion solution to reach the sampling site at the end of the outlet tube.

Two perifusion solutions were employed. The first consisted of HBSS without glucose to which bovine albumin fraction V [Sigma] 500 mg per 100 ml and D-glucose 30 mg per 100 ml were added. This solution was utilized during the first 40 minutes of perifusion to allow stabilization of the islet beta cells at 37°C with no stimulus for insulin release. At 40 minutes, the intake tubing was transferred to the second solution containing the same salt solution with the same concentration of albumin but having a D-glucose concentration of 300 mg per 100 ml to provide a stimulus for insulin secretion. These perifusion solutions were maintained at 37°C in a waterbath. Their initial pH was adjusted to 7.35-7.40 with bicarbonate and, since they tended to become alkalotic,

FIGURE 5. Perifusion apparatus.

1 - 37°C waterbath

2 - Perifusion solution

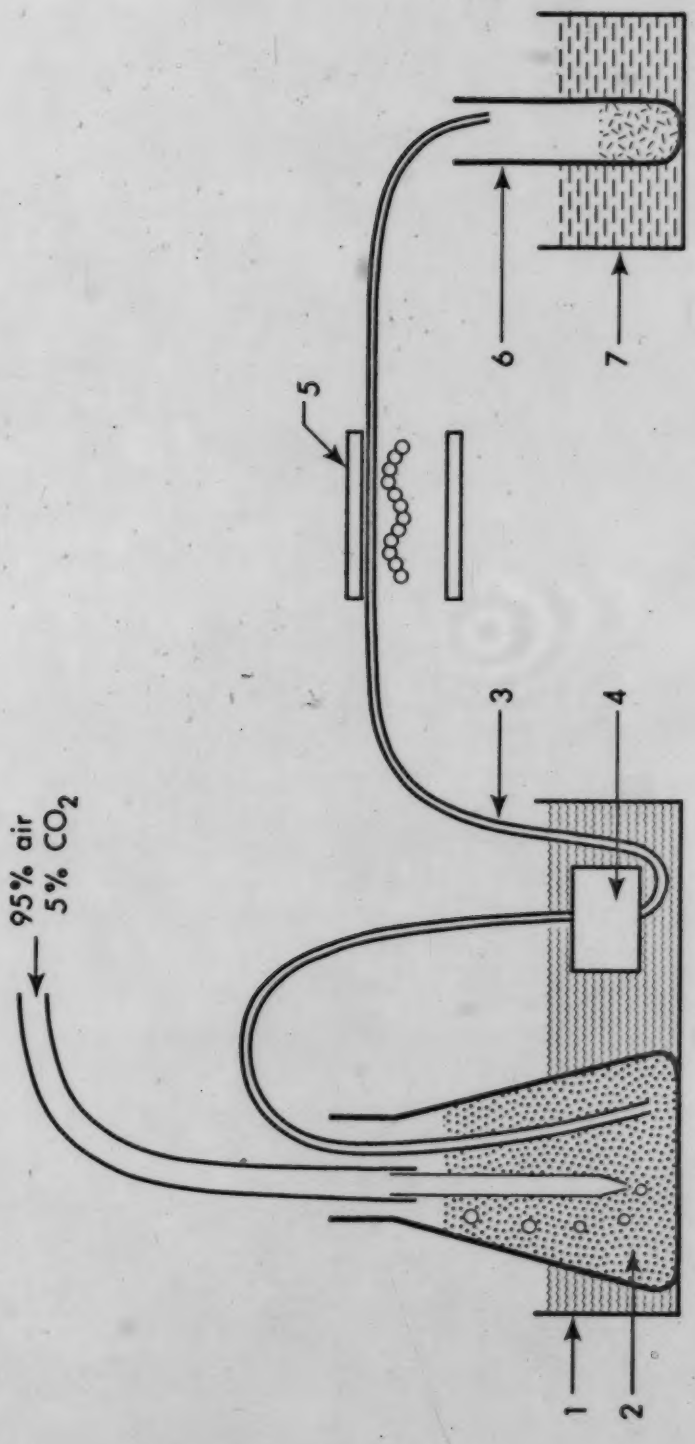
3 - Silastic tubing

4 - Filter chamber containing isolated islets

5 - Peristaltic pump

6 - Polystyrene tube

7 - Ice bath

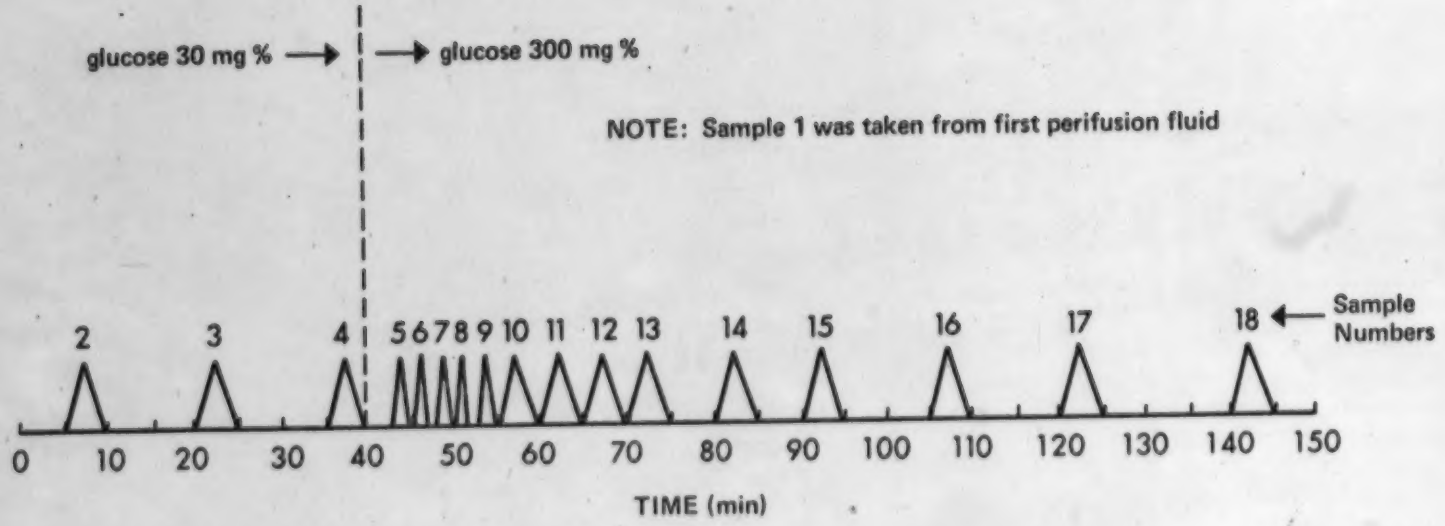

95% air
5% CO_2
1
2
3
4
5
6
7

the pH was maintained at 7.35-7.45 by bubbling a 95% air-5% CO_2 mixture through the solution. The pH was monitored intermittently with a Fisher Accumet pH meter and the flow rate of the gas mixture adjusted accordingly.

For each study, isolated islets from two of the experimental groups were perifused in parallel. To place the islets on the millipore filters the inlet tubing was removed and a glass 10 ml syringe inserted in the intake. Using a large bore pipette, the islets in Dextran from the harvest procedure were transferred to the syringe. Three 10 ml volumes of HBSS without glucose at 4°C were syringed through the filter and outlet tubing to flush the islets onto the filter, wash away the Dextran, and remove all air from the system. The intake tubing was filled with HBSS without glucose and returned to its proper position on the filter with its free end in the first perifusion fluid. The islet holding filter was submerged in the 37°C waterbath and perifusion was begun.

An aliquot of the first perifusion solution was taken directly from the flask to determine background insulin concentration in the solutions. Samples of the perifusion fluid from the outlet tubing were collected according to a previously determined time sequence [see footnote] to demonstrate the insulin secretory response over a total perifusion period of 2 hours and 25 minutes as shown in Fig. 6. Three samples collected during perifusion with the first solution were intended to determine the baseline rate of unstimulated insulin secretion. As noted above, the first sampling after changing to the second perifusion solution was delayed for 3 minutes to compensate for the dead space

FIGURE 6. Sampling sequence during *in vitro* perifusion of islets.


glucose 30 mg %
glucose 300 mg %
NOTE: Sample 1 was taken from first perifusion fluid
2
3
4
5 6 7 8 9 10 11 12 13
14
15
16
17
18
Sample Numbers
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
TIME (min)

within the system. Samples were then collected for 2 minutes each at closely spaced intervals during the early post-stimulation period to attempt to demonstrate increased insulin secretion rates as early as possible. During the later more stable stages of insulin secretion, samples were collected for 5 minute periods at more widely spaced intervals. A total of 18 samples were obtained for each perifusion experiment. All samples were collected on ice in 6 ml polystyrene tubes and immediately frozen for subsequent insulin assay.

Following the termination of perifusion, the filters were removed from the chambers and flooded with a 1% solution of Neutral Red in HBSS. After 10 minutes, the excess stain was washed off with HBSS and the individual islets of Langerhans could be counted under a dissecting microscope at 15 times magnification. The individual islets are distinguishable as distinct, globular, intensely red stained bodies in contrast to the irregular shaped less deeply stained acinar tissue and the tubular ductal elements. Counting was performed independently by two persons and the results were averaged and brought to the nearest multiple of 5.

3. Insulin Assay

The amounts of insulin in the samples from the perifusion experiments were determined by a modification of the radioimmunoassay technique of Hales and Randle (35) utilizing the reagents and minor modifications of the protocol of the Schwarz/Mann Insulin Radioimmunoassay Kit.

The principle of this technique is that insulin antibody may be pre-precipitated with anti-gamma globulin serum while retaining its

ability to react with insulin. This allows for separation of antibody-bound insulin from free insulin by centrifugation which can precipitate the complex. By incubating an unknown sample and a known quantity of I^{125}-labelled insulin with a limited number of specific antibody binding sites, a competitive reaction occurs. The level of radioactivity [labelled insulin] present in the antigen-antibody complex isolated following the incubation is inversely related to the concentration of insulin in the unknown sample. The actual concentration of insulin in the unknown is determined by comparing its specific radioactivity to a standard curve prepared by adding various known levels of unlabelled insulin to the appropriate quantities of labelled insulin and antibody binding sites.

The reagents used in the assay were:

(a) unknown samples

(b) human insulin standard at a concentration of 200 μU/ml

(c) I^{125} labelled porcine insulin at a concentration of 1.25 ng/ml, labelled with .125 μCi I^{125}

(d) insulin binding reagent which is porcine insulin antiserum raised in guinea pigs and precipitated with anti-guinea pig serum from rabbits

(e) 0.04 M phosphate buffer with bovine albumin fraction V [Sigma] 500 mg per 100 ml.

The assay was performed in 12×75 mm polystyrene tubes to which the reagents have shown no binding affinity. All aliquoting of reagents was performed with the Oxford Sampler Micropipetting system.

There were three steps in the assay:

1) The initial stage combined unlabelled human insulin standard or unknown samples with the binding reagent and an appropriate amount of phosphate buffer to make the volume of the reaction mixture approximately 200 µl. The volumes added are shown in Table I. Tube 1 determined the background radioactivity of this system. Tubes 2 through 9 were for constructing a standard curve and were performed in triplicate. Tube 10 represents the unknown samples from perifusion which were performed in duplicate. Each assay involved 200 tubes consisting of 4 background, 24 standards, and 172 unknowns. These reaction mixtures were incubated for 6 hours at 4°C as a short preincubation period before adding labelled insulin has been shown to increase the sensitivity of the assay.
2) Following the 6-hour incubation, 100 µl of I^{125} labelled porcine insulin was added to each tube. The tubes were then placed in a refrigerated Packard Gamma Scintillation Spectrometer, model 578, at 4°C for a further incubation period of 18 hours and duplicate counting of each tube to obtain "Total Counts Per Minute [CPM]" for each sample.
3) After 18 hours, 1 ml of phosphate buffer was added to each tube and agitated with a vortex mixer. Centrifugation at 4500 g for 20 minutes at 4°C in a Servall RC-2 refrigerated centrifuge was used to precipitate the antigen-antibody complexes. The supernatant was decanted gently and discarded. The tubes were returned to the gamma spectrometer and counted three times to obtain "Residual CPM".

Tube No.	Insulin Content (μU)	Unknown (μl)	Insulin Standard (μl)	Binding Reagent (μl)	Phosphate Buffer (μl)
1	0	-	-	-	200
2	Trace	-	-	100	100
3	0.4	-	2	100	100
4	1.0	-	5	100	100
5	2.0	-	10	100	100
6	3.0	-	15	100	100
7	5.0	-	25	100	100
8	10.0	-	50	100	50
9	20.0	-	100	100	-
10	Unknown	25	-	100	100

TABLE 1: Volumes of reagents combined during first step of insulin radioimmunoassay.

At the completion of these three steps, Total and Residual CPM values were known for each tube. The Residual CPM for tube 1 represents the background radioactivity of the assay and the average result of the four samples was subtracted from both the Total and Residual CPM values of each of the other tubes to obtain "Corrected" CPM values. For each tube, the percentage of labelled insulin fixed to the binding reagent was calculated by dividing the Corrected Residual CPM by the Corrected Total CPM and multiplying by 100. That is:

$$\text{Percent Bound} = \frac{\text{Corrected Residual CPM}}{\text{Corrected Total CPM}} \times 100$$

To construct the standard curve, the percent bound was plotted against the known standard insulin values [μU/tube] for the triplicate samples of tubes 2 to 9 on semilogarithmic graph paper with μU/tube as the log function.

From the percent bound values calculated for the unknown samples, tube 10, the amount of insulin [μU/tube] in each could be read from the standard curve. Having added 25 μl, which is 1/40 ml, of unknown sample to each tube, multiplying the μU/tube value by 40 calculated the insulin concentration [μU/ml]. The final concentration value for each unknown was an average of the duplicate assay performed for each.

4. Interpretation of Results

The experimental procedures outlined give four basic results for each perifusion experiment:

a) the number of islets of Langerhans

b) the flow rate of the perifusion system

c) the background insulin concentration in the perifusion solutions

d) the insulin concentration in the effluent perifusion fluid samples collected at specific time intervals.

From these values, a common basis for analysis of the experiments can be reached by calculating the "Insulin Secretion per Islet per Minute" at each sampling time using the formula:

$$\text{Insulin secretion } (\mu U/\text{islet/min}) = \frac{[\text{Total insulin conc.} - \text{Background insulin conc. } (\mu U/ml)] \times \text{Flow rate } (ml/min)}{\text{Number of Islets}}$$

A qualitative impression of the insulin secretion response can be gained by graphically displaying the perifusion response with the insulin secretion values plotted on the ordinate versus sample time on the abscissa. This can be done both for individual experiments and for the mean secretion values of each experimental group.

Statistical analysis of the results can be approached in two ways:

a) The mean and standard error values for insulin secretion at each sampling period for each experimental group can be determined and statistical comparison between groups for each sampling period can be performed using the unpaired Student's t-test. This method determines differences in magnitude of the insulin secretory response on a temporal basis.

b) By calculating the area beneath the curve of the insulin secretory response graph following glucose stimulation,

"Total Insulin Secretion per Islet" can be calculated for each experiment. This determination involves summation of calculations of insulin secretion between successive sampling times using the formula:

$$\text{Total insulin secretion } (\mu U/\text{islet}) = \frac{\text{Insulin secretion time}^{n}+\text{time}^{n+1}\ (\mu U/\text{islet/min})}{2} \times [\text{time}^{n+1} - \text{time}^{n}\ (\text{min})]$$

The sample time was considered to be that at which the collection was completed. For each experimental group, mean and standard error values of total insulin secretion can be determined and groups compared by the unpaired Student's t-test, achieving a comparison based solely on the magnitude of the response.

Footnote:
Sampling Sequence During Perifusion

In order to demonstrate the biphasic nature of the insulin secretory response during perifusion samples must be collected at frequent intervals, resulting in such a large number of samples that each experiment is very costly due to the expense of insulin radioimmunoassay. Furthermore, a degree of variability in the first phase of secretion often results in it being obscured when the mean secretion values of several experiments are calculated. Therefore, as the *in vitro* studies of islet insulin secretion following ischemia were primarily interested in the presence and degree of the secretory response rather than its dynamics, the number of samples taken was limited to that which would adequately display the overall secretory response but not its biphasic nature.

C. Results

The original experimental design was to demonstrate the insulin secretory capability of islets of Langerhans harvested from animals exposed to increasingly prolonged warm ischemic intervals, if necessary for up to several hours, until a time was reached when no secretory response occurred. Such a time would be assumed to correlate with the end-point of viability of the islets for transplantation. The initial experiments therefore undertook to isolate and perifuse islets from animals subjected to 0 [control], 1/2, 1, 2, and 3 hours of warm ischemia.

It became apparent that with increasing ischemia fewer islets were being isolated and that beyond 1 hour no appreciable numbers of islets could be harvested. It therefore became important to quantify how many islets could be isolated from control and ischemic animals. In the perifusion experiments, a range for number of islets perifused of 30 to 200 had been chosen so as to have enough islets to produce detectable amounts of insulin but not so many as to exceed the upper limit of the insulin assay. If by visual inspection there had appeared to be a large number of islets in the Dextran gradient, not all were transferred to the perifusion apparatus and the final count did not represent the total number of islets isolated from each animal. Therefore a second series of experiments was carried out in which islets were harvested from groups of 10 animals exposed to 0, 1/2, 1, and 2 hours of warm ischemia and immediately counted. The number of islets isolated after 0, 1/2, and 1 hour are shown in Table 2 and Figure 7.

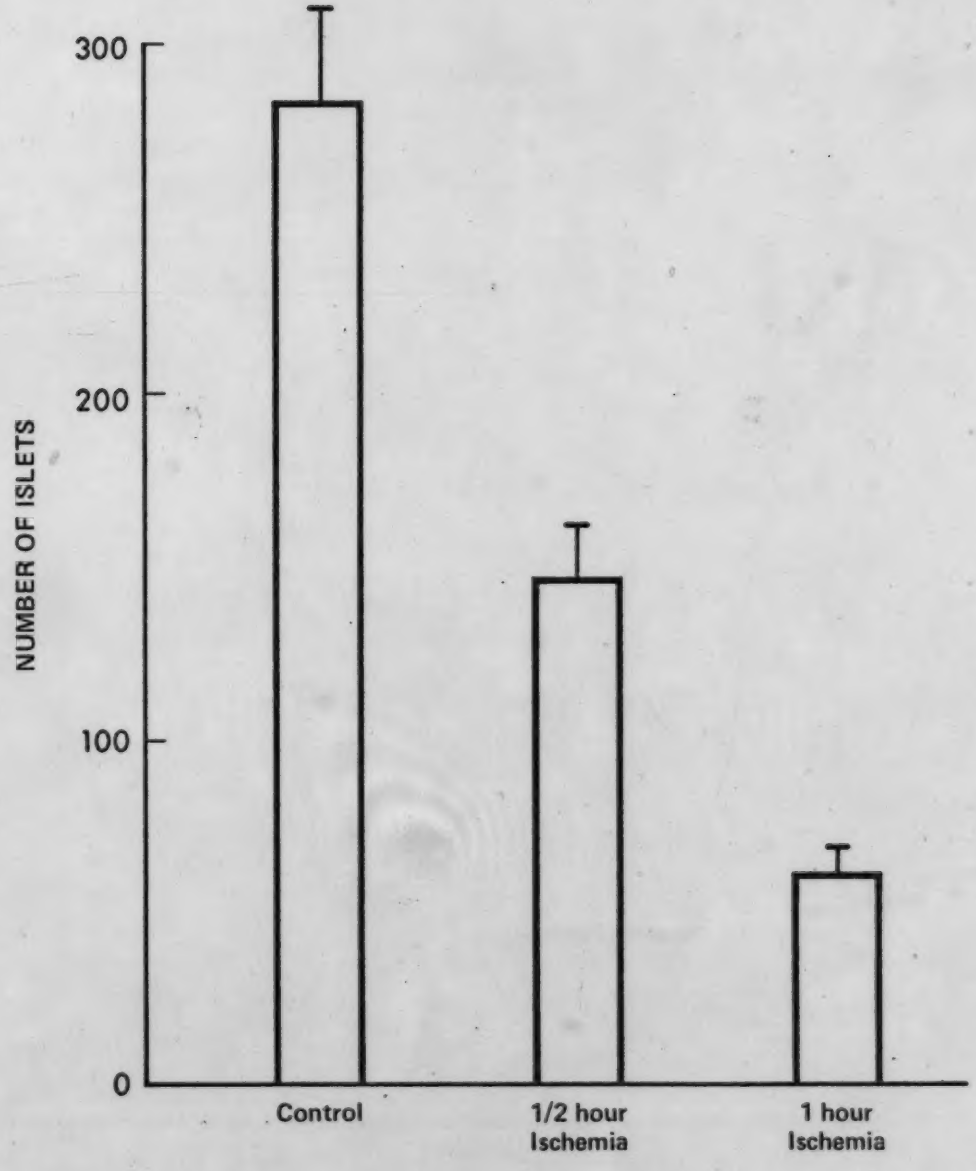
The differences in yield at each successively prolonged period of ischemia showed a statistically significant decrement in the number

Ischemic Interval	Number of Islets		
0 (Control)	283±29	p<0.001	p<0.001
1/2 hour	147±17	p<0.001	
1 hour	62±7		

TABLE 2: Number of islets isolated from control and ischemic animals.

Values are Mean±1 Standard Error.

FIGURE 7. Number of islets isolated from control and ischemic animals.


300
200
100
0
NUMBER OF ISLETS
Control
1/2 hour
Ischemia
1 hour
Ischemia

of islets that could be recovered. Following 2 hours of ischemia, there was no visual evidence of islet tissue at the appropriate interfaces in the Dextran density gradient. When these layers were transferred to the filter paper and stained, the few objects which due to their structural appearances may have been isolated islets often stained poorly, making their positive identification difficult. In most instances there were less than 10 structures which could be tentatively identified as islets. Modifications of several aspects of the isolation procedure - collagenase concentration, digestion time, concentration of Dextran solutions in the density gradient - did not improve the harvest yield from additional animals subjected to 2 hours of warm ischemia. Therefore, perifusion studies were limited to 0, 1/2, and 1 hour of ischemia.

The *in vitro* insulin secretory responses of islets from the experimental groups during perifusion are tabulated in Table 3 and displayed graphically in Figure 8. The control group began to increase their rate of insulin secretion within 7 minutes of being challenged with the high glucose concentration and developed a rapid rise in rate until at the time of terminating perifusion a plateau of secretion at 2.29 μU/islet/min was being approached, which was greater than 4 times the baseline level of 0.53. Insulin release from ischemic islets continued to decline towards the baseline following glucose stimulation for 12 minutes in the 1/2 hour group and 20 minutes in the 1 hour group, suggesting that perhaps a longer stabilization period of approximately 1 hour would have been useful in establishing a more definite baseline secretory rate. The 1/2 hour group then began a definite insulin

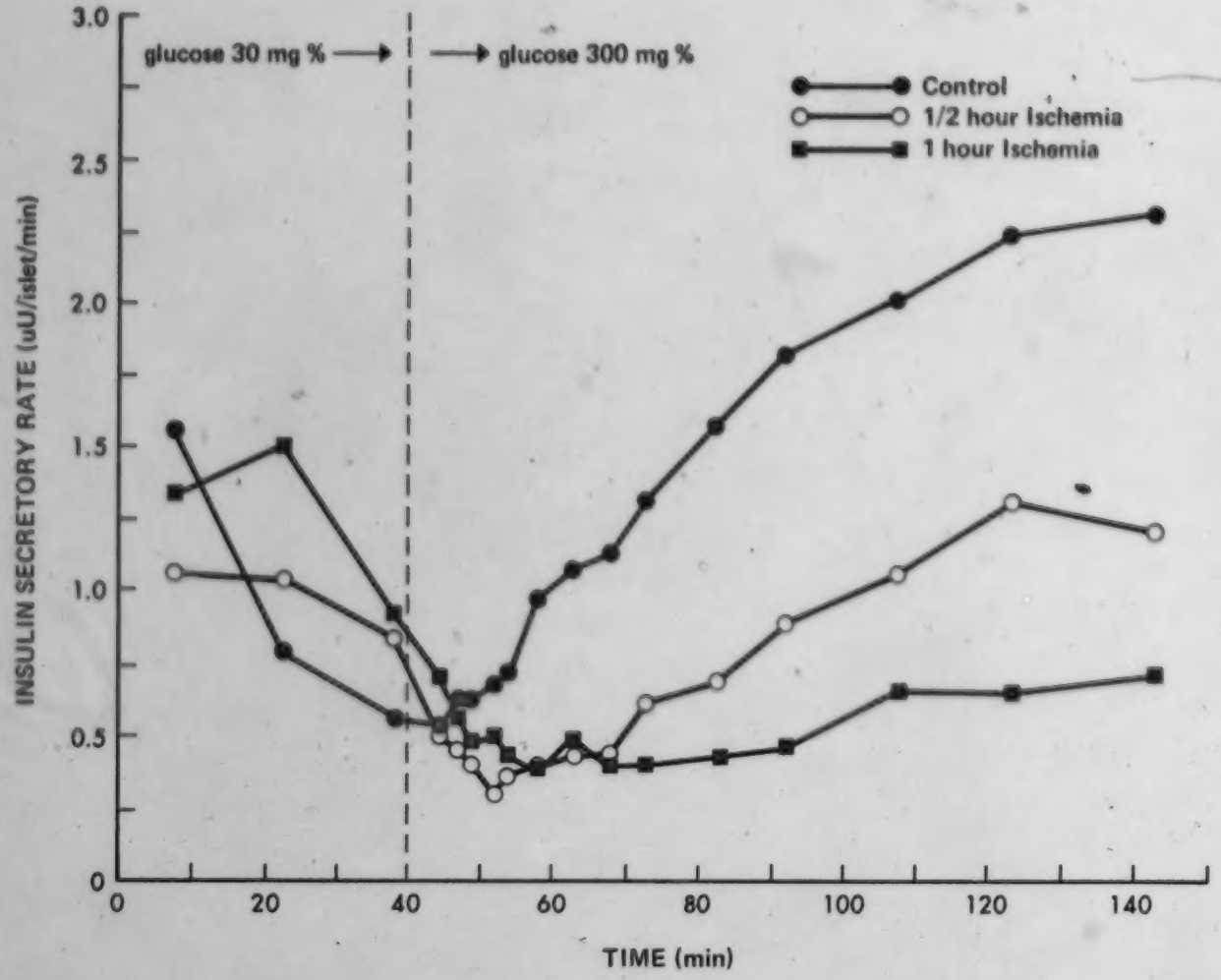
Sample No.	Control	1/2 hr Ischemia	1 hr Ischemia
2	1.53±0.31	1.07±0.35	1.33±0.19
3	0.80±0.12	1.05±0.27	1.51±0.23
4	0.56±0.08	0.83±0.25	0.94±0.11
5	0.53±0.07	0.51±0.13	0.71±0.10
6	0.62±0.10	0.46±0.12	0.56±0.07
7	0.62±0.09	0.40±0.10	0.47±0.05
8	0.66±0.10	0.30±0.05	0.50±0.08
9	0.71±0.12	0.37±0.07	0.44±0.06
10	0.97±0.15	0.40±0.04	0.37±0.04
11	1.08±0.17	0.44±0.03	0.50±0.07
12	1.12±0.16	0.45±0.03	0.40±0.05
13	1.32±0.23	0.62±0.08	0.41±0.09
14	1.58±0.28	0.68±0.08	0.43±0.06
15	1.83±0.27	0.90±0.10	0.45±0.09
16	2.01±0.26	1.07±0.11	0.67±0.09
17	2.23±0.26	1.32±0.15	0.65±0.10
18	2.29±0.27	1.21±0.10	0.71±0.10

TABLE 3: *In vitro* insulin secretion rates of control and ischemic islets during perifusion.

Values are Mean±1 Standard Error

Units are µU insulin/islet/min.

FIGURE 8. *In vitro* insulin secretion rates of control and ischemic islets during perifusion.


glucose 30 mg %
glucose 300 mg %
Control
1/2 hour Ischemia
1 hour Ischemia
INSULIN SECRETORY RATE (uU/islet/min)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
120
140
TIME (min)

secretory response but the slope of the response curve was less steep than for the control group, reaching a maximum rate of 1.32 μU/islet/min at 120-125 minutes of perifusion, and declining to 1.21 at the end of perifusion. The 1 hour ischemia group showed a very delayed and slow rise in detected insulin secretory rate, reaching a maximum rate at the termination of perifusion of 0.7 μU/islet/min which is less than twice the baseline rate. This data demonstrated a marked progressive deterioration in the insulin secretory capabilities of islets from animals exposed to 1/2 and 1 hour of warm ischemia as compared to those harvested from living animals. The rate of secretion by the ischemic groups was significantly [$p<0.05$] less than for the control group at all sampling times beyond 55 minutes of perifusion. The secretory rate of the 1 hour ischemia group is significantly decreased compared to the 1/2 hour group at 80 minutes of perifusion and beyond.

The deterioration of islet function was confirmed by examining their total insulin secretion during the high glucose stimulation period of perifusion [Table 4, Fig. 9]. The total secretion showed a significant progressive reduction for each successive experimental group. The impairment of total secretion after 1 hour was highly significant [$p<0.001$] when compared to the control group.

D. Discussion

The results of this study indicate that there can be little optimism that islets of Langerhans which will have satisfactory post-transplantation function can be harvested from cadavers following exposure to warm ischemia.

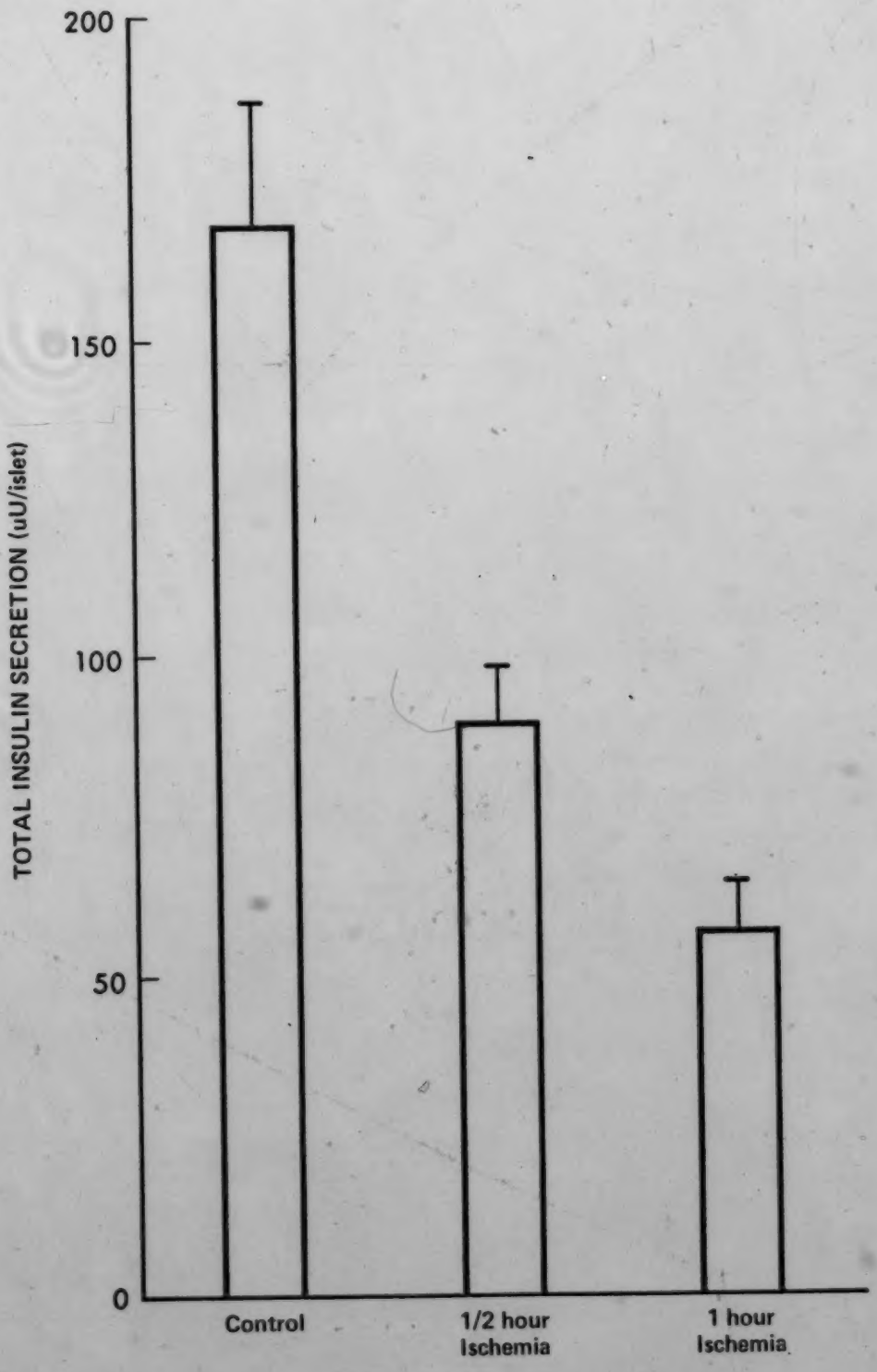
The rapid decline in the numbers of islets which can be isolated

Ischemic Interval	Total Secretion (μU insulin/islet)		
0 (Control)	167.76±21.95	p<0.01	p<0.001
1/2 hour	89.10±8.41	p<0.02	
1 hour	58.84±6.68		

TABLE 4: Total insulin secretion of control and ischemic islets during perifusion.

Values are Mean±1 Standard Error.

FIGURE 9. Total insulin secretion of control and ischemic islets during perifusion.


200
150
100
50
0
TOTAL INSULIN SECRETION (uU/islet)
Control
1/2 hour Ischemia
1 hour Ischemia

after ischemic intervals, leading to a total lack of success in obtaining them from animals subjected to longer than one hour of ischemia, greatly reduces the efficiency and feasibility of transplanting islets from post-mortem donors. Obviously, even brief periods of ischemia are altering the islets in some manner so that the harvest procedures used in these experiments are no longer productive. When the low percentage yield of the present rigorous isolation procedures in control animals is considered, it is not surprising that any additional insult to the donor tissue, such as the metabolic changes resulting from ischemia, leads to complete inability of the islets to withstand these procedures. It is conceivable that this phenomenon is due to damage secondary to ischemia, perhaps even early autolysis, affecting the delicate reticular network surrounding the islets so that they are less able to maintain their integrity upon exposure to the harsh mechanical and/or enzymatic methods for their isolation. Perhaps newer harvest techniques or pre-treament of the donor animal with protective drugs may increase the ischemic interval following which islets can be isolated in useful numbers. Without such advances, attempts to obtain transplantable islets from cadaver donors would appear to be impractical.

The *in vitro* function studies demonstrated that ischemia rapidly results in diminished insulin secretory activity of isolated islets. If the ability to secrete insulin does indeed correlate with islet viability as has been proposed in this report, the experimental data indicates that a significant impairment of viability has occurred following 1/2 hour of ischemia and the limited response at one hour is suggestive that the end-point of viability has been nearly reached at

that time.

The metabolic defects whereby decreased islet secretory function evolved were not studied in this project but possible mechanisms can be considered. Decreased insulin content of the islets during ischemia due to its passive loss from the beta cells plus arrest of synthesis during that interval would make available appreciably less insulin for active secretion. However, as normally in the later stages of insulin secretion newly synthesized hormone is being released, intact cell metabolism in ischemic islets should have produced at least a late increase in secretory rate. Alternatively, impairment of the pathways of insulin secretion at any point - glucose metabolism, ATP generation, calcium transport, microtubule contraction - would arrest secretion. However, interruption of one cell process which is particularly susceptible to ischemia may not represent cell death. Therefore, the *in vitro* perifusion model may be too sensitive a test of islet function to equate with viability as it may be detecting the acute interruption of some cellular functions following ischemia and harvest procedures. The impairment demonstrated might be reversible under favorable conditions such as culture in nutrient media or the post-transplantation environment.

The viability of ischemic islets might be demonstrated to be more prolonged by examination of more fundamental cell metabolism. However, on the basis of these studies of insulin secretory ability, very poor success in reversing diabetes mellitus could be expected following transplantation of islets from donors subject to any period of warm ischemia, especially in excess of one hour.. Support for the

validity of the functional criteria of islet viability established by this research is suggested by their similarity to the parameters of ischemic tolerance of other transplantable organs.

Kidney viability following ischemia has received considerable attention due to its practical applications in clinical renal transplantation. Employing canine models of occluding the renal vascular pedicle with (18) or without (39, 43) subsequent autotransplantation of the kidney, plus contralateral nephrectomy, the maximum period of ischemia tolerated has been shown to be 75 minutes (18), "approximately one hour" (43), and "between one and two hours" (39). Adhering to a 60-minute time restriction for warm ischemic exposure, a satisfactory success rate can be achieved in human renal transplantation from cadaver donors (10, 36).

The warm ischemic tolerance of the liver is not precisely defined due to the recognition that different aspects of heptic metabolism are affected at varying times and a determinant factor of cell death has not yet been selected. A significant reduction in hepatic adenine nucleotide levels, particularly ATP and ADP, plus a marked release of lysosomal enzymes, LDH, SGOT, SGPT, and alkaline phosphatase within 30 minutes of ischemia have been demonstrated (27, 30). The degree of change in these parameters correlates with prognosis for survival. Van Wyk *et al* (34) studied *in vitro* hepatic function [O_2-uptake, bromsulphalein clearance, ammonium clearance, ATP generation] in pigs following *in situ* ischemia and observed only a modest reduction in function at 30 minutes but severe impairment at 60 minutes and beyond from which they concluded that satisfactory hepatic function cannot be

expected if organ harvesting is delayed beyond 1/2 hour after death. Observing survival of dogs after hepatic circulatory occlusion *in vivo*, several investigators have proposed the duration of ischemia compatible with viability to be 75 (24), 50 (42), and 40 minutes (27). Following auxiliary hepatic transplantation, Toledo-Pereyra *et al* (82) found the survival of livers exposed to 30 minutes of warm ischemia to be less than six days.

Islets of Langerhans do not appear more resistant to ischemia than other organs such as kidney and liver. In addition to the metabolic dysfunction resulting from ischemia, alterations in physical integrity occur which interfere with their isolation by current technqiues.

CHAPTER IV

IN VITRO FUNCTION OF ISLETS OF LANGERHANS SUBJECTED TO WARM ISCHEMIA FOLLOWING DONOR PRETREATMENT WITH METHYLPREDNISOLONE

A. Introduction

Due to the well recognized adverse effects of warm ischemia on the function and survival of organs for transplantation, proven means of reducing ischemic injury, such as hypothermia and *in vitro* perfusion, have become integral parts of organ preservation programs. In the continuing search for additional methods, a popular field of investigation has been pharmacologic pretreatment of the donor prior to death to attempt to reduce autolysis [corticosteroids], maintain vascular integrity [heparin, vasodilators] and preserve cellular energy stores [allopurinol]. Therefore, a logical extension of the foregoing functional studies of islet of Langerhans tolerance to ischemia was to determine if donor pretreatment was of any benefit in protecting them from ischemia. Investigation in this project was limited to corticosteroids because vascular integrity is not important in islets as they are not implanted by vascular anastomosis and an intravenous preparation of allopurinol was not available at the time this research was undertaken.

One of the major pathophysiologic processes in cellular disruption during ischemia is the initiation of intracellular autophagy by the release of lysosomal hydrolases (23, 28, 30, 79). Corticosteroids

have been shown to delay the release of enzymes from lysosomes, probably by a stabilizing effect on their limiting membranes, following many insults (86) including ischemia (28, 79), thereby theoretically retarding autodigestion. Application of this theory to the ability of organs to withstand ischemia has shown positive results in the survival of animals following episodes of hepatic (29) and renal (17, 60) ischemia which are lethal to control animals.

The potent and popular corticosteroid, methylprednisolone, was chosen for use in these experiments. The pretreatment regimen was derived from the work of Miller and Alexander (60) who, by varying dosage and pretreatment interval for kidneys prior to ischemia, obtained optimum results with 30 mg/kg methylprednisolone administered intravenously for a period of two hours.

B. Materials and Methods

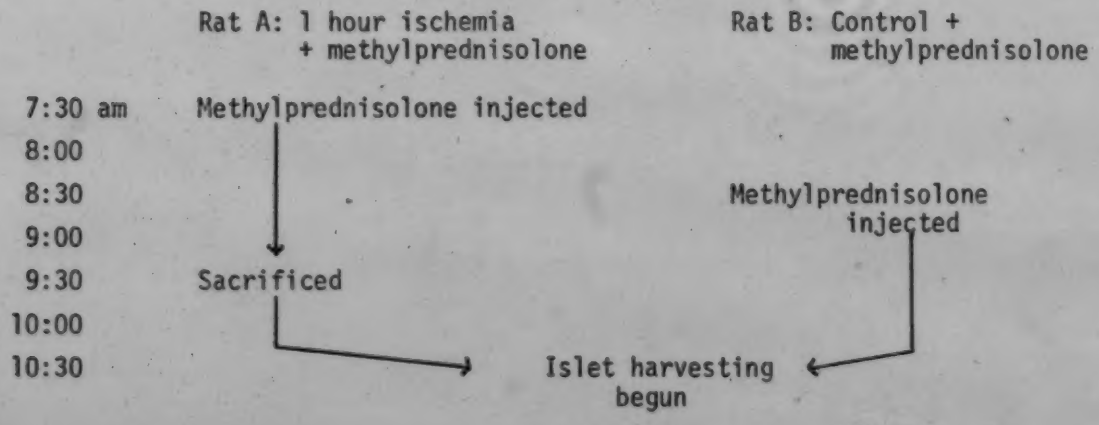
The methylprednisolone pretreatment study assessed the insulin secretory capability of islets harvested from male Wistar rats given methylprednisolone 30 mg/kg intravenously two hours prior to beginning to harvest islets in the control group or sacrificing the animals in the study groups for a period of warm ischemia. Experimental groups of ten animals were subjected to 0 [control], 1/2, and 1 hour of warm ischemia following the pretreatment interval. All experiments were parallel perifusions of islets isolated from one animal from two of the different groups.

All techniques of islet isolation, perifusion, and insulin radioimmunoassay were the same as for the warm ischemia without pretreatment study. The additional methods required to administer

the methylprednisolone pretreatment were:

1. Inferior Vena Cava Cannulation

Within one week prior to drug administration, an indwelling cannula was placed in the inferior vena cava. This route for giving the methylprednisolone was chosen because it provided reliable access to the venous system with none of the time delays or faulty injections due to technical problems which can arise with acute methods such as tail vein puncture or femoral vein cannulation. The predictable success of intravenous injections was important as careful timing of injection relative to the beginning of islet harvesting was required, particularly as these were parallel studies of animals from two experimental groups. A typical sequence of injection and harvesting in a paired experiment would be:



All cannulations were performed under ether anesthesia. The ventral abdomen and a 2 cm square area on the dosum of the neck were shaved and washed with an iodine solution. A 5-6 cm midline lower abdominal incision was performed and the inferior vena cava exposed just above the juncture of the common iliac arteries. The overlying

peritoneum and soft tissues were bluntly pushed aside with a sponge to expose approximately 2 cm of the vena cava and the right psoas muscle. A 4-0 silk suture was then placed in the right psoas muscle just caudal to the planned vena cava cannulation site and tied, both ends of the suture being left long for subsequent tying around the cannula to secure it in place. A PE 50 polyethylene cannula had been previously prepared by filling an approximately 25 cm length with normal saline, sealing one end by compression with heated forceps, and cutting the opposite end on an approximately 45° angle. The cannula was introduced into the vena cava by puncturing the anterior wall with the sharpened end and it was advanced 2 to 3 cm cephalad within the lumen. The previously placed suture was then tied tightly about the cannula and a second anchoring suture was placed in similar fashion approximately 1 cm distally. The caudally directed free end of the cannula was then formed into a wide loop through the abdominal cavity, the loop being secured to the left anterior abdominal wall with a single suture of 4-0 silk, and brought subcutaneously at the cephalad end of the incision. The linea alba was closed with a continuous suture of 3-0 chromic. A blunt probe was then passed to the right subcutaneously from the superior end of the incision to the prepared area on the dorsum of the neck and its leading end delivered through a skin stab wound. The free end of the cannula was then secured in a longitudinal slit at the trailing end of the probe, allowing it to be placed in the subcutaneous tunnel as the probe was withdrawn through the stab wound. The cannula was secured to the stab wound with a suture ligature of 2-0 silk and any excess length was excised after resealing the lumen with a heated forcep, leaving a

2 cm segment exposed. The ventral skin incision was closed with a continous horizontal mattress suture of 4-0 silk.

This procedure had a mortality rate of less than 10% with deaths due to anesthetic errors or uncontrollable bleeding upon insertion of the inferior vena cava cannula. Usually there was essentially no blood loss and the animals recovered uneventfully. Postoperatively they were housed in individual cages to prevent them dislodging each others cannulae.

2. Administration of Methylprednisolone

The desired dosage of methylprednisolone [Solu-Medrol, Upjohn] was 30 mg/kg. Therefore, 20 mg of methylprednisolone was dissolved in 1.5 ml sterile distilled water so that each of the animals, whose mean weights were approximately 250 g, received an injection volume in the range of 0.4 ml.

To administer the drug, the awake animals were immobilized securely by hand, the heat sealed end of the cannula was cut off, and a 25 gauge needle was inserted in its lumen. Sterile normal saline [0.5 ml] was injected to check for free flow via the cannula, the appropriate volume of the methylprednisolone solution was injected, and finally the cannula was again flushed with 0.5 ml saline so that all the drug was carried into the venous system. The needle was removed and the cannula resealed with heated forceps. The animal was then returned to its cage for its pretreatment interval of two hours before being sacrificed for a period of ischemia or anesthetized for harvesting of control islets.

C. Results

As this study was undertaken following completion of the perifusion experiments on non-pretreated islets, it was anticipated that the problem of being able to harvest fewer islets from ischemic animals would also be encountered. Therefore the total number of islets that could be isolated was obtained from the same series of animals in which perifusion studies were performed, with the exception of one pair of experiments in which this step was inadvertently omitted. This simply required either complete transferring of the islets from the Dextran gradient to the perifusion apparatus so that all would be counted at the termination of perifusion or, if by visual inspection more than 200 islets were estimated to be present in the gradient, an appropriate number were taken for perifusion and the remainder transferred to a separate filter, stained, and counted so that a total count could be obtained. The number of islets harvested from each methylprednisolone pretreated group plus comparison to those isolated from the corresponding non-pretreated groups are shown in Table 5 and Figure 10.

These results indicate that a similar trend was present with progressive inability to isolate islets from animals exposed to increasingly prolonged periods of warm ischemia regardless of whether or not methylprednisolone pretreatment had been administered. The animals subjected to 0 [Control] and 1/2 hour of ischemia yielded very similar numbers of islets in both series of experiments. Following 1 hour of ischemia, significantly more islets were recovered from the methylprednisolone pretreatment group, indicating that there may be some benefit of steroids in improving the yield of islets after that period of

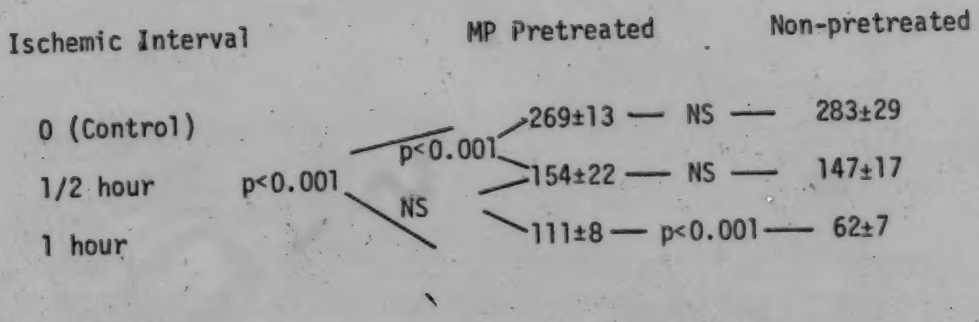
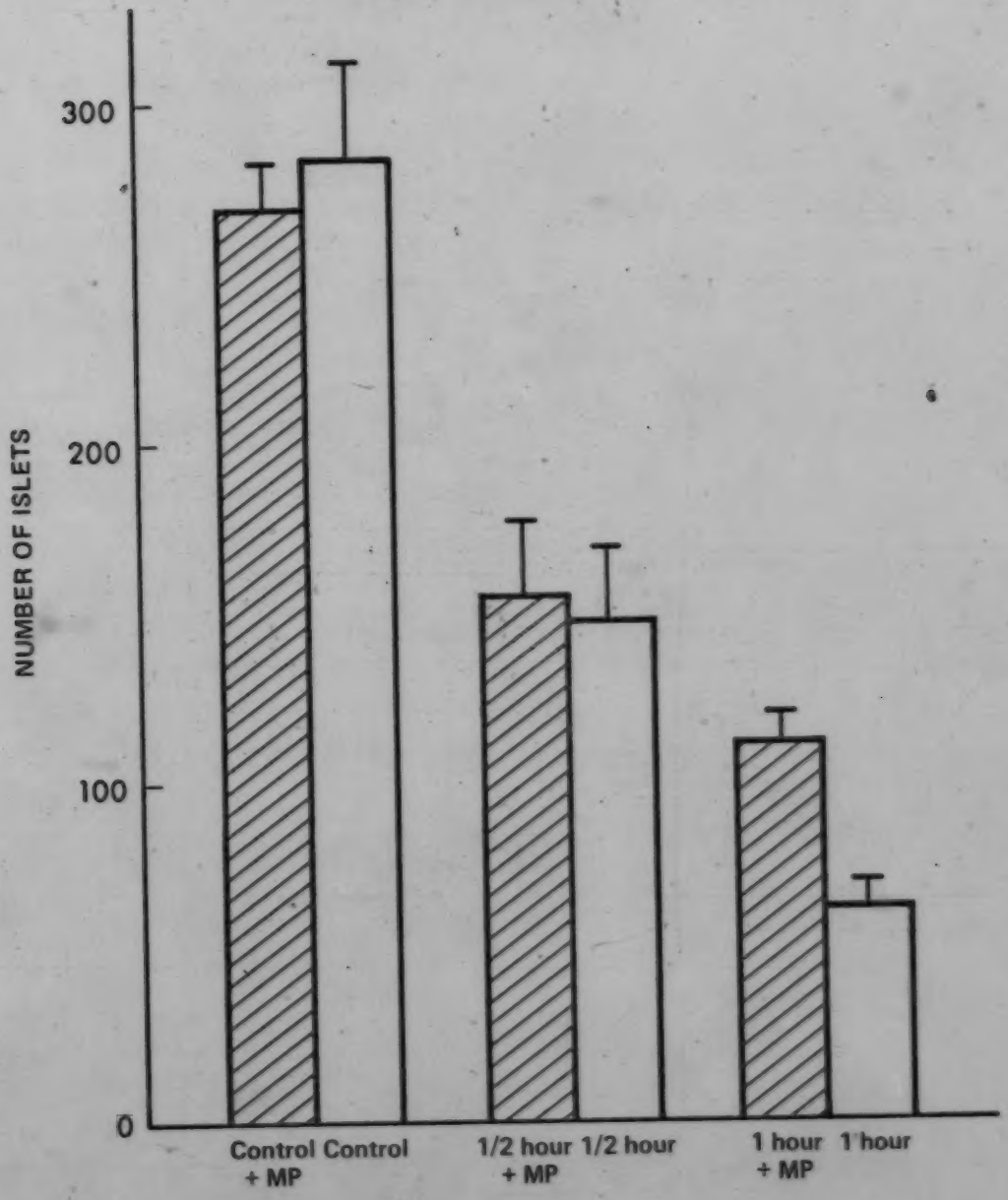


TABLE 5: Number of islets isolated from methylprednisolone (MP) pretreated and non-pretreated animals.

Values are Mean±1 Standard Error.

NS - not significantly different.

FIGURE 10. Number of islets isolated from methylprednisolone (MP) pretreated and non-pretreated animals.


NUMBER OF ISLETS
300
200
100
0
Control
Control + MP
1/2 hour
1/2 hour + MP
1 hour
1 hour + MP

ischemia. However, several attempts were also made to isolate islets from animals subjected to 2 hours of ischemia following methylprednisolone pretreatment and, as in the non-pretreatment study, no appreciable number of positively identifiable islets could be recovered at that time.

The *in vitro* insulin secretory function of islets from the groups of animals pretreated with methylprednisolone is shown in Table 6 and Figure 11. All groups continued to demonstrate diminishing insulin secretion for 7 minutes following stimulation with the high glucose concentration. Thereafter, the control group developed a moderate insulin secretory response, reaching a maximum secretory rate of 1/27 μU/islet/min at 120-125 minutes of perifusion and declining slightly to 1.22 at the end of perifusion, which is approximately 3 1/2 times the baseline rate of secretion. The 1/2 and 1 hour groups showed very similar secretory responses with a very gradual increase in secretory rate to little more than twice the baseline rates. At no sampling period was there a statistically significant difference in secretory rates between the 1/2 and 1 hour groups. The control group secretory rate was significantly [$p<0.05$] greater than the 1/2 hour group at 80 minutes and beyond and greater than the 1 hour group from 60 minutes to the termination of perifusion.

The secretory responses of methylprednisolone pretreated and non-pretreated islets are compared in Figure 12. The control and 1/2 hour ischemic groups from the methylprednisolone pretreatment study had impaired insulin secretion. For the control group the impairment was significant [$p<0.05$] from 80 minutes of perifusion onwards; for the

Sample No.	Control+MP	1/2 hr Ischemia+MP	1 hr Ischemia+MP
2	2.06±0.46	1.92±0.26	1.28±0.18
3	0.68±0.10	0.84±0.07	0.92±0.09
4	0.45±0.07	0.61±0.09	0.67±0.09
5	0.48±0.13	0.48±0.06	0.44±0.05
6	0.41±0.08	0.38±0.07	0.37±0.05
7	0.36±0.06	0.31±0.07	0.32±0.05
8	0.40±0.06	0.36±0.07	0.35±0.05
9	0.53±0.08	0.33±0.09	0.36±0.05
10	0.56±0.09	0.34±0.09	0.37±0.05
11	0.64±0.07	0.39±0.10	0.39±0.05
12	0.73±0.10	0.43±0.11	0.38±0.06
13	0.82±0.13	0.55±0.15	0.38±0.05
14	0.95±0.10	0.55±0.15	0.46±0.05
15	1.14±0.11	0.67±0.15	0.51±0.07
16	1.16±0.12	0.76±0.13	0.59±0.09
17	1.27±0.11	0.77±0.16	0.69±0.10
18	1.22±0.11	0.82±0.15	0.70±0.11

TABLE 6: *In vitro* insulin secretion rates of methylprednisolone (MP) pretreated islets.

Values are Mean±1 Standard Error.

Units are μU insulin/islet/min.

FIGURE 11. *In vitro* insulin secretion rates of methylprednisolone (MP) pretreated islets during perifusion.

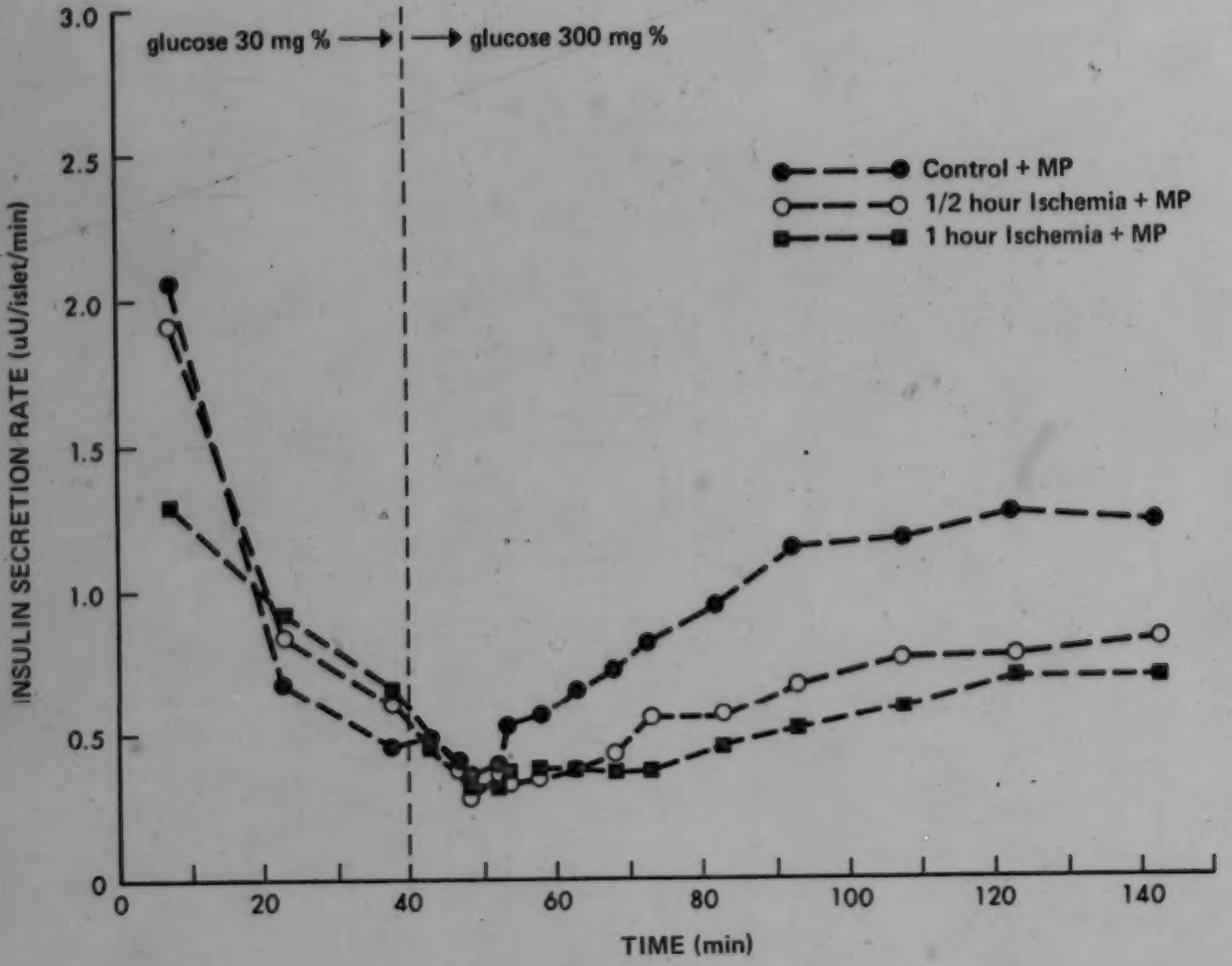
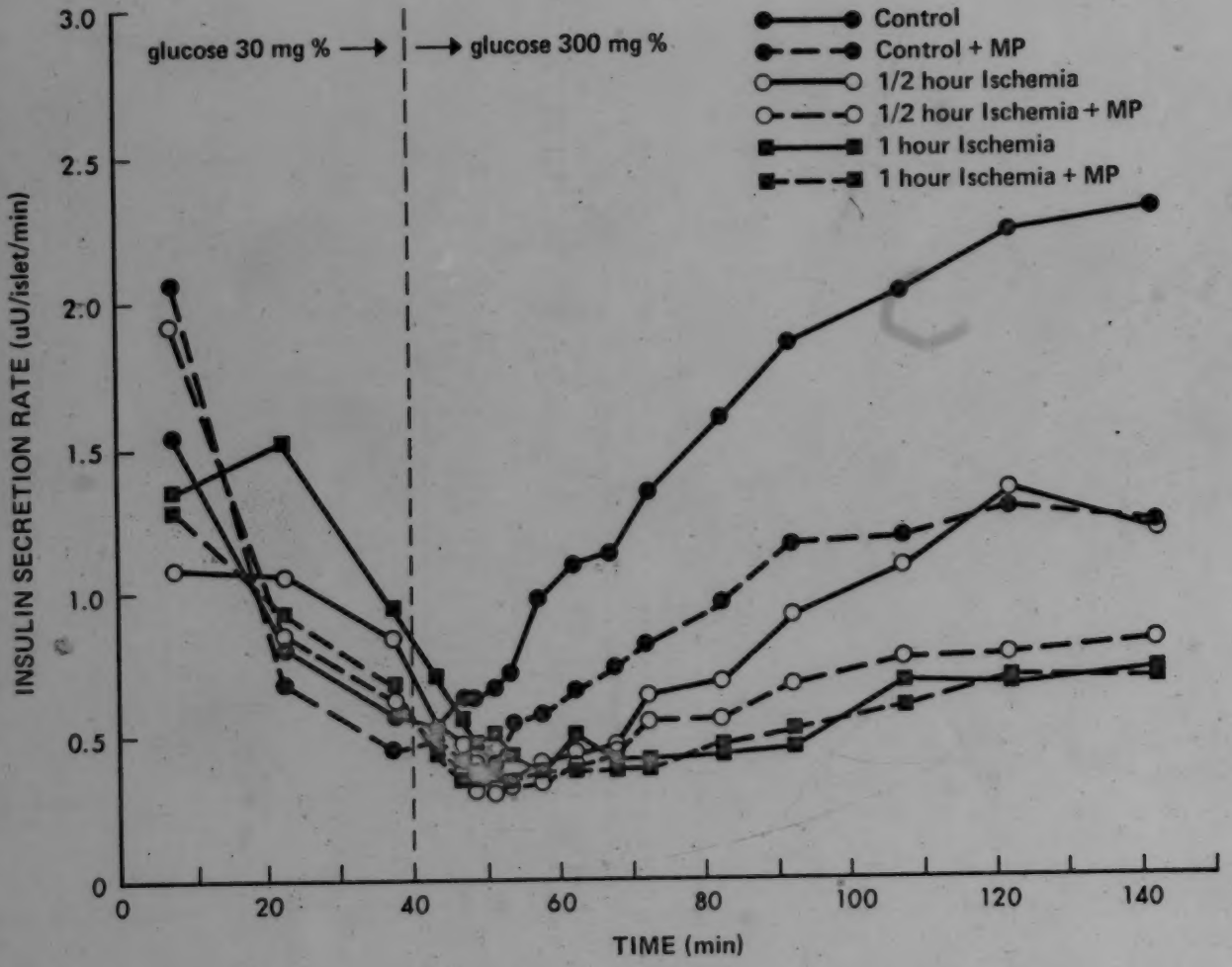

glucose 30 mg %
glucose 300 mg %
Control + MP
1/2 hour Ischemia + MP
1 hour Ischemia + MP
INSULIN SECRETION RATE (uU/islet/min)
TIME (min)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
120
140

FIGURE 12. *In vitro* insulin secretion rates of methylprednisolone (MP) pretreated and non-pretreated islets during perifusion.


glucose 30 mg %
glucose 300 mg %
Control
Control + MP
1/2 hour Ischemia
1/2 hour Ischemia + MP
1 hour Ischemia
1 hour Ischemia + MP
INSULIN SECRETION RATE (uU/islet/min)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
120
140
TIME (min)

Ischemic Interval		MP Pretreated		Non-pretreated
0 (Control)		98.63±8.74	p<0.01	167.76±21.95
1/2 hour	p<0.01 / p<0.05	64.04±12.90	NS	89.10±8.41
1 hour	p<0.01 / NS	54.78±7.04	NS	58.84±6.68

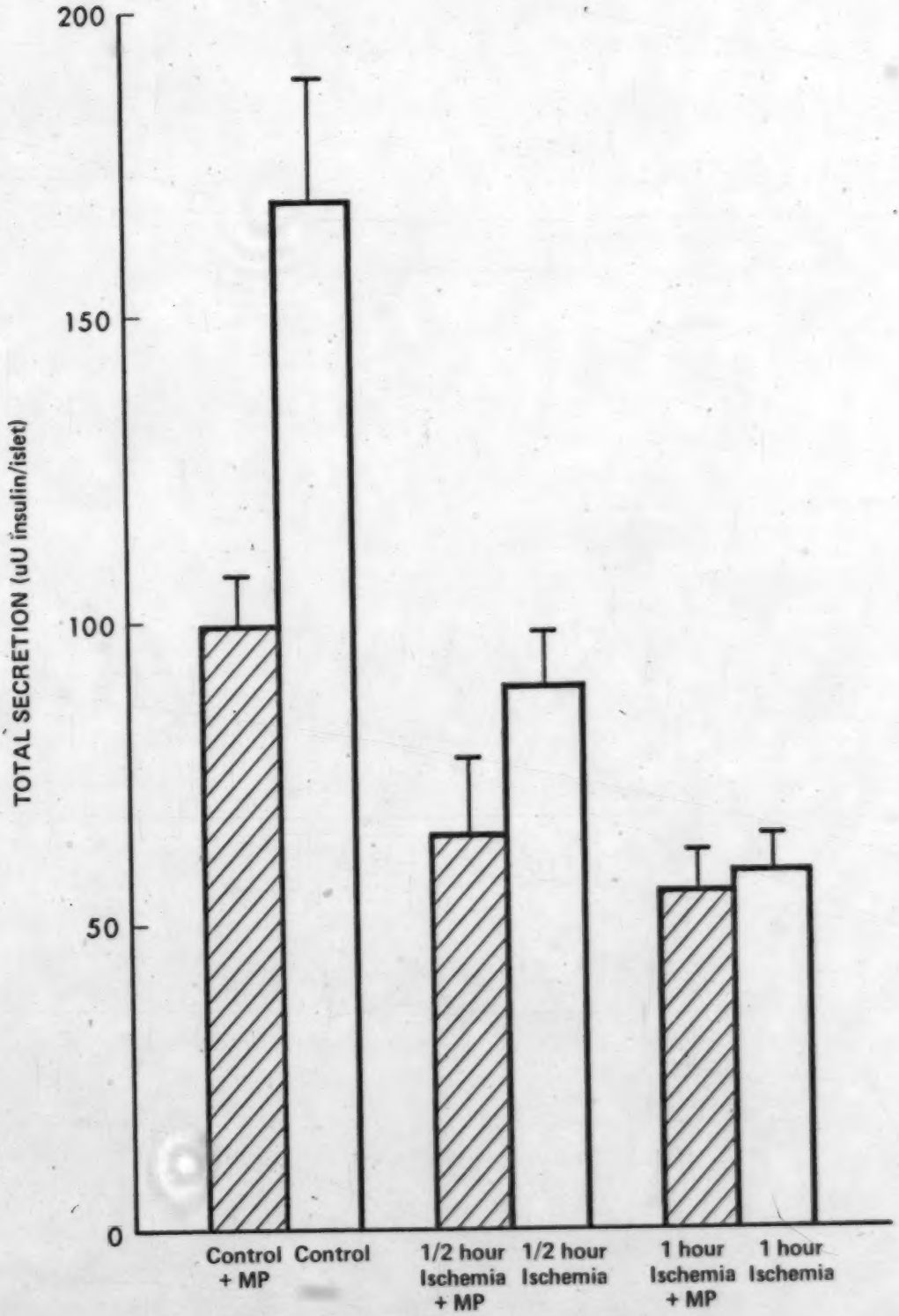
TABLE 7: Total insulin secretion of methylprednisolone (MP) pretreated and non-pretreated islets during perifusion.

Values are Mean±1 Standard Error.

Units are μU insulin/islet.

NS - not significantly different.

FIGURE 13. Total insulin secretion of methylprednisolone (MP) pretreated and non-pretreated islets during perifusion.


200
150
100
50
0
TOTAL SECRETION (uU insulin/islet)
Control + MP
Control
1/2 hour Ischemia + MP
1/2 hour Ischemia
1 hour Ischemia + MP
1 hour Ischemia

1/2 hour group a significant reduction in response was present at 120 minutes and beyond. The 1 hour ischemia groups from both studies displayed almost identical secretory responses. Insulin release from the 1/2 hour ischemia, methylprednisolone pretreatment group was also very similar to that of the 1 hour groups.

The total insulin secretion following glucose stimulation for the three experimental groups from the pretreated and non-pretreated studies is given in Table 7 and Figure 13. As in the non-pretreated study, total insulin secretion by islets from animals receiving methylprednisolone pretreatment showed a progressive decline at successive ischemic intervals, even though the difference between 1/2 and 1 hour is not significant. The reduction, compared to the non-pretreated groups, was significant for the control group and showed a definite but non-significant trend in the 1/2 hour ischemia group. Total secretion by the two 1 hour groups was very similar.

D. Discussion

The results of this study, within the limits to be proposed, suggest that little benefit was derived from methylprednisolone pretreatment of islet donors prior to warm ischemia.

The decreasing numbers of islets isolated following each ischemic interval was quite comparable to the results of the non-pretreatment study. A beneficial effect of methylprednisolone in increasing the number of islets harvested was recognized at one hour of ischemia but did not lead to any prolongation of the ischemic interval following which islets could be isolated. Furthermore, the significant increase in number of islets harvested at one hour still resulted in

significantly fewer than from the nonischemic groups, indicating that delayed harvesting would continue to be an inefficient source of islets for transplantation.

The impaired *in vitro* insulin secretion of the control and 1/2 hour ischemia groups of islets from methylprednisolone pretreated in comparison to the non-pretreated study, with very similar patterns of secretion by the one hour groups from both studies, can possibly be explained as follows.

The two projects were undertaken consecutively with the methylprednisolone pretreatment study begun following completion of perifusion experiments on non-pretreated islets. Therefore, different lots of reagents, particularly Dextran and collagenase, were employed as former stocks were used up. Altered chemical constitution of the different lots may have had an effect on the physiologic function of islets isolated during their use [73, Appendix II]. Perhaps the marked similarity of responses of the one hour groups in each study provide some reassurance that such results might not have been achieved had a toxic reagent been used in one series of experiments.

An alternative explanation might be that methylprednisolone pretreatment changed the response of the beta cells to glucose stimulation. As steroids are not considered to be toxic to cells, it can probably be assumed that such an effect would have been due to physiologic alteration of the insulin secretory mechanism.

Glucocorticoids, of which methylprednisolone is a relatively potent representative, promote gluconeogenesis and hepatic glycogenesis and impair peripheral utilization of glucose (37, 61). Species

variation in the predominance of each of these effects leads to either normoglycemia [dogs, most humans] or varying degress of hyperglycemia [sheep, rabbits, rats] during the chronic administration of pharmacologic doses of steroids (4, 11, 31, 56, 85). In all species studied, an increase in serum insulin levels accompanies glucocorticoid administration. The hyperinsulinism is attributed to peripheral resistance to glucose utilization plus a possible increased sensitivity of the beta cells to glucose in normoglycemic subjects, and to the additional stimulus of elevated blood glucose levels in hyperglycemic animals. No studies documenting the rapidity of change in serum glucose and insulin concentrations with the acute administration of steroids were found.

An attractive hypothesis would be that intravenous administration of massive doses of glucocorticoid as in these pretreatment experiments would rapidly induce gluconeogenesis, mobilizing glucose into the circulation to cause a secondary stimulation of insulin release. Direct steroid stimulation of the beta cells might also occur. The net result would be reduced total insulin content of the islets following steroid administration, as has been confirmed by demonstrations of beta cell degranulation by electron microscopy (85) and a reduction in total extractable pancreatic insulin (12). The provoked insulin secretion would also consume substrates for insulin synthesis. The reductions in islet insulin content and substrates for de novo insulin synthesis would limit the insulin secretory response that could occur during *in vitro* perifusion.

This theory of rapid glucocorticoid induced insulin release might therefore explain the reduced secretory response in the control

and 1/2 hour ischemic methylprednisolone pretreated islets. Its effect probably would not have been apparent in the one hour ischemic islets due to the limited insulin secretion occurring at that time.

Possible alterations in insulin homeostasis due to the metabolic effects of methylprednisolone introduces a second variable making comparison of viability based on the insulin secretory rates of ischemic islets with pretreatment to those without impossible. However, it is tempting to suggest that the very limited response in both one hour ischemia groups implies a similar severe degree of impairment of viability.

Insulin secretory rate cannot be used as an index of islet viability following donor pretreatment if the drugs employed have any effect on glucose homeostasis, particularly the beta cell insulin content.

CHAPTER V

CONCLUSIONS

These studies of islet of Langerhans structural and functional integrity following exposure to warm ischemia permit the following conclusions.

1. Light microscopy failed to demonstrate any morphologic changes in islets following up to 24 hours of warm ischemia. The lack of information from the histologic techniques used and their failure to correlate with electron microscopic and *in vitro* functional evaluations of islets following ischemia indicate that this method is valueless in assessing islet of Langerhans viability for transplantation.

2. Electron microscopy demonstrated a sequence of deterioration in islet ultrastructure during warm ischemia whereby irreversible morphologic damage, defined by the disruption of cell and organelle limiting membranes, could be recognized by six hours. In view of the probably more accurate indication of viability by subsequent *in vitro* functional studies, the limit of viability determined by electron microscopy was too generous, significant functional impairment having preceded irreversible structural disintegration.

3. *In vitro* functional studies exposed two limiting factors in

obtaining islets from postmortem donors.

a) Increasing warm ischemic intervals were associated with a progressive reduction in the number of islets that could be harvested. Beyond one hour of ischemia, virtually none could be isolated by current techniques.

b) *In vitro* insulin secretory function rapidly deteriorated during ischemia; only a very limited response occurred after one hour. This functional deterioration can be extrapolated to indicate a comparable impairment of viability.

4. Methylprednisolone pretreatment of islet donors for *in vitro* functional studies was not found to be beneficial.

a) A positive effect in increasing the number of islets harvested following one hour of ischemia failed to produce numbers comparable to those from nonischemic donors and did not lead to prolongation of the ischemic interval during which islets could be isolated.

b) *In vitro* islet function was adversely affected by methylprednisolone in the control and 1/2 hour ischemia groups while the function of the one hour group was comparable to its non-pretreatment counterpart. This functional impairment may be attributed to a pharmacological effect of methylprednisolone or an uncontrollable variation in experimental procedure. It emphasizes that *in vitro* studies of islet viability based on insulin secretory function following donor pretreatment are unreliable if the pretreatment drugs have any effect on islet homeostasis.

The practical application of these findings in rats is to suggest guidelines for islet harvesting in human transplantation. The maximum warm ischemic period following which any number of islets with any degree of *in vitro* function can be isolated is one hour. To obtain significantly better results in numbers of islets harvested and their function demands that the warm ischemic interval be kept to a minimum. With current methods, cadaver donors exposed to periods of warm ischemia will be an unsatisfactory source of islets of Langerhans for transplantation.

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APPENDIX I

WARM ISCHEMIA
TEMPERATURES TO WHICH ISCHEMIC ISLETS WERE EXPOSED

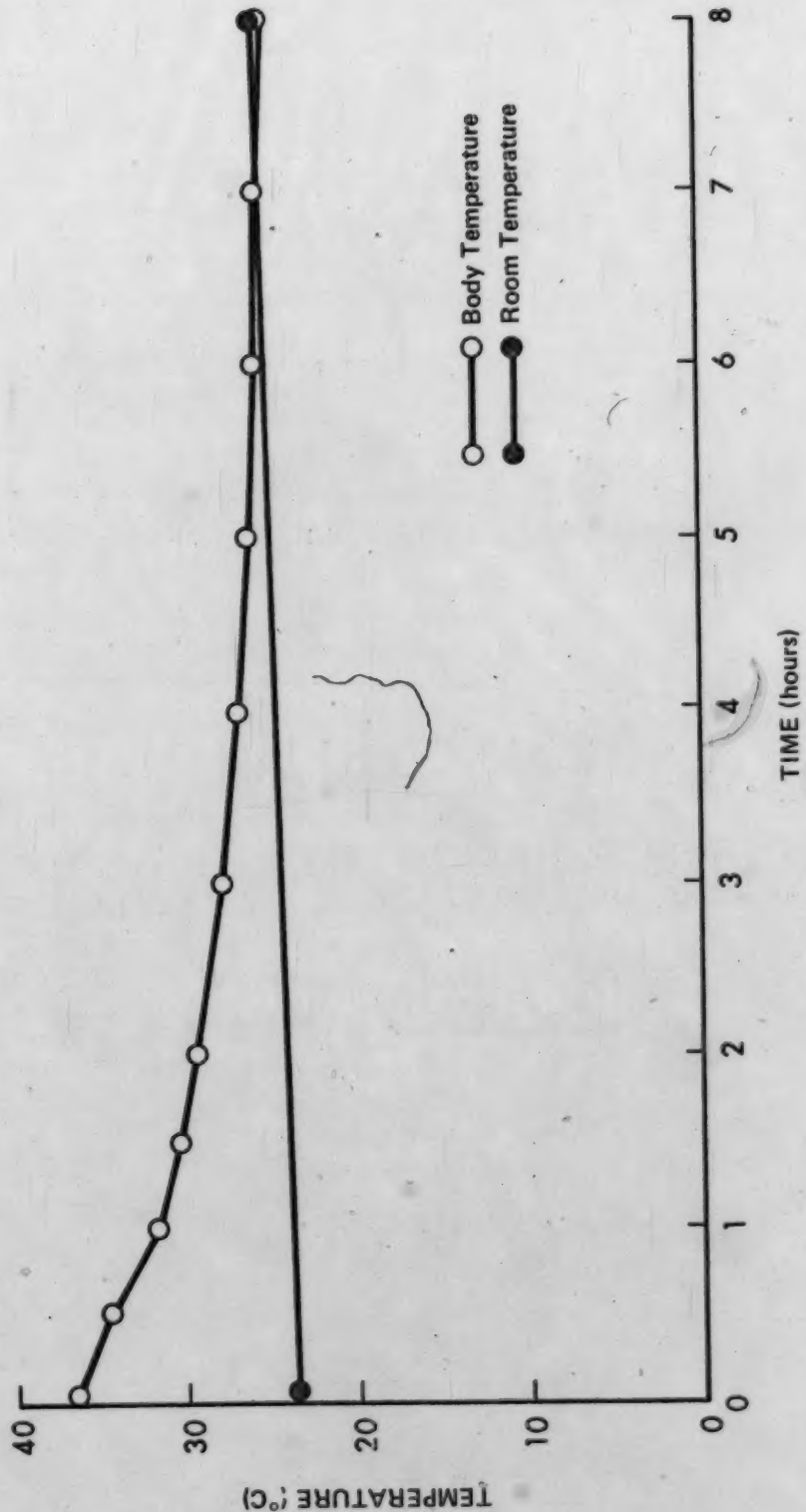
Warm ischemia may be defined as the interval between the interruption of normal circulation and the beginning of effective cooling or artificial perfusion. Due to the complex sequence of degenerative intracellular events which are initiated by ischemia, this interval is critical to the subsequent recovery of cellular and organ function. The rate at which ischemic injury evolves is temperature dependent. The rate of metabolic deterioration occurring at normothermia can be slowed considerably by cooling to room temperature and to a very significant degree by hypothermic conditions [4°C].

All studies described in this report were designed to mimic the warm ischemic conditions of harvesting organs from cadaver donors. Each animal to serve as a donor of ischemic tissue was sacrificed by an overexposure to ether, had its abdomen shaved for eventual laparotomy, and was then left exposed at room temperature [22-26°C] for the appropriate ischemic interval prior to removing the pancreas for experimentation.

To document the temperatures to which the islets were exposed *in situ* in the rat cadavers, five Wistar rats of similar size range to those used in the actual experiments were sacrificed and prepared in the usual manner. Immediately after death a pointed fine calibre temperature probe was inserted percutaneously through the ventral abdominal wall and positioned blindly so that its recording tip would

be estimated to lie anterior to the lumbar spine in the region of the pancreas. This probe was connected to a continuous temperature recorder [Varian Model G-11A] on which an initial room temperature reading had been previously obtained. The animal remained connected to the apparatus until its internal body temperature had equilibrated with room temperature. The final room temperature was then noted. The pattern of body cooling, which was very similar in all animals, is shown in Figure 14. The gradual cooling of the animal to room temperature between seven and eight hours after death indicates that the islets were subject to an appreciable period of warm ischemia, particularly in reference to the time intervals during which significant injury was demonstrated by electron microscopy and *in vitro* functional studies.

FIGURE 14. Postmortem body temperature in rats.


Body Temperature
Room Temperature
TEMPERATURE (°C)
TIME (hours)
0
10
20
30
40
0
1
2
3
4
5
6
7
8

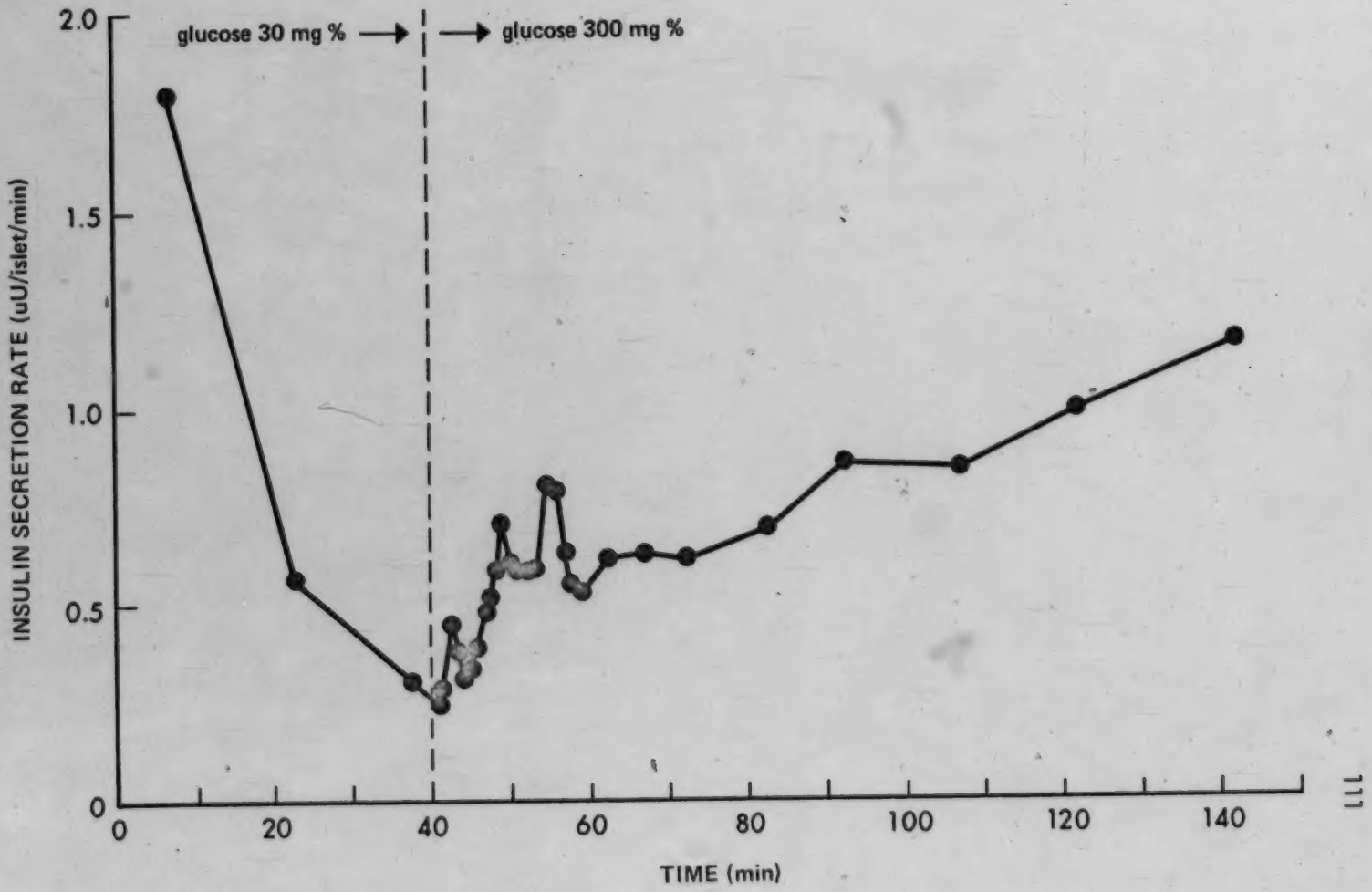
APPENDIX II

USE OF DEXTRAN FOR DENSITY GRADIENT ISOLATION OF ISLETS

Ficoll, a high molecular weight polymer of sucrose, has been the reagent reported exclusively for use in constructing density gradients for the isolation of islets of Langerhans. Scharpe *et al* (73) have shown that different lots of commercially available Ficoll are contaminated to varying degrees with low molecular weight substances which appear to be toxic to islets. Reproducible results of isolated islet insulin secretory responses or transplantation are impossible to obtain unless these contaminants are removed from the Ficoll by dialysis prior to its use.

As facilities for dialyzing Ficoll were not available for this project, it was thought that clinical grade Dextran, a high molecular weight polymer of dextrose which has been used successfully in other density gradient separation techniques, might be sufficiently pure for use. Density gradients constructed of Dextran, average molecular weight 170,000, achieved separation of islets from acinar tissue equally effectively as those prepared with Ficoll. The effect of Dextran on islet insulin secretion was assessed by perifusion experiments in which samples of the effluent fluid were collected every minute during the initial 20 minutes following stimulation with a high glucose concentration [300 mg%] and for longer and more widely spaced intervals during the later stages of stimulation. The dynamics of insulin secretion demonstrated in a typical experiment are shown in Figure 15. The

FIGURE 15. Biphasic insulin secretory response during perifusion following islet isolation with Dextran.


glucose 30 mg %
glucose 300 mg %
INSULIN SECRETION RATE (uU/islet/min)
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
120
140
TIME (min)

biphasic nature of the insulin secretory response is recognizable with the initial rapid phase represented by two small peaks of secretion within the first 15 minutes followed by the more gentle slope of the sustained second phase of release.

The adequate separation of islets plus the satisfactory insulin secretory responses during perifusion appeared to justify the use of Dextran for islet isolation in place of undialyzed Ficoll.

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